Tributyltin: Advancing the Science on Assessing Endocrine Disruption with an Unconventional Endocrine-Disrupting Compound

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Contents

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1 Introduction

Tributyltin (TBT) was introduced as a biocide in the 1960s and today its use is widely restricted by a variety of statutes. Although some countries (and local governments within those countries) restricted the use of TBT in antifouling paints as early as 1982 (Alzieu 2000; Champ 2000), it was not until 2008 that a more global ban was enacted. In that year, the International Convention on the Control of Harmful Antifouling Systems for Ships required its signatories to ensure that vessels would no longer use hull paint containing TBT or other organotin chemicals. This agreement was signed by 74 countries as of the end of October 2016. Unfortunately, a recent study has confirmed that recreational vessels sampled from countries around the Baltic Sea still contain high concentrations of TBT and triphenyltin (TPT) and may be a source to the environment (Lagerström et al. 2016).

The main inputs of tributyltin into the environment are contaminated water and sediment originating from ports, harbors, marinas, and boat yards due to leaching from boat paint and improper disposal. Contaminated sediment can be mobilized by dredging, bioturbation, ship scour, or weather events and TBT-contaminated water can be carried by currents to previously unimpacted locations. Tributyltin may also be introduced into the environment by ongoing release from previously treated

structures (continuous release and during cleaning/renovation) and small-scale (mis-)use or disposal of prohibited antifouling products. Diffuse exposure may also arise from the use or disposal of previously TBT-treated wooden articles and other applications including its use as a stabilizer for PVC products, antifungal treatment, and as a preservative for wood, paper, and textiles (US EPA 2003).

Tributyltin may be one of the most toxic man-made chemicals ever intentionally released into the environment, eliciting endocrine-type responses at concentrations in the range of 1 ng/L for water or 10 ng/g for whole body. Even concentrations in the range of low μg/L aqueous or low μg/g tissue cause high rates of mortality in many species. However, outright mortality events due to TBT exposure are relatively rare. Therefore, the most important environmental consequences result from sublethal responses. Among them, the development of male sexual characteristics in female marine gastropods exposed to TBT, a phenomenon known as imposex, has been abundantly documented (e.g., Gibbs and Bryan 1996a). This abnormality has resulted in reproductive failure of populations of Caenogastropods globally, leading to mass extinction and subsequent alterations in community structure and functioning of coastal ecosystems (Gibbs and Bryan 1986, 1996b; Hawkins et al. 1994).

The main goal for this review was to examine the available literature on TBT as an EDC and provide a synopsis on population-relevant responses across major taxa. Additionally, we highlight the case of TBT as an unusual endocrine disruptor and discuss some of the reasons why its toxic potential and MeOA went unrealized for many years. Finally, we use the Organization for Economic Co-operation and Development (OECD) Conceptual Framework for Endocrine Disruptor Testing and Assessment to organize the available information on effect assessment and environmental exposure levels to conduct a tentative retrospective environmental risk assessment of TBT.

2 Methods

2.1 Literature Search and Selection of Data

To conduct this review, 160 references were selected from an initial list of approximately 965 regulatory reports and open and grey literature, in an attempt to capture relevant data from original studies on fish (45 references), molluscs (55 references), and other taxonomic groups including mammals (60 references); these were sorted according to the type of effect. This was not intended to be an exhaustive review of the extensive TBT literature, so it is possible that some relevant studies were inadvertently omitted.

2.2 Quality Evaluation of Relevant Data

TBT is a data-rich compound with numerous tests at various levels of biological organization. However, the vast majority of these studies did not follow

international standardized test guidelines. Nevertheless, several full or partial life-cycle tests are available for mammals, fish, and several invertebrate taxa. Data previously validated for regulatory reviews (e.g., EU 2005; US EPA 2003, 2008) were assumed reliable; however, as far as possible, original studies were used as sources of data. Other ecotoxicity studies were quality checked and scored using Klimisch scores (e.g., using the ToxRTool: https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/toxrtool). Only studies ranked as Klimisch 1 and 2 were used for the subsequent analysis (although a small number of papers ranked as Klimisch 3 or 4 were used as supporting information if their findings were verified by other studies). However, Klimisch scores do not apply to field studies, which were a major source of data. Similarly, histopathological investigations are difficult to evaluate using these criteria. Therefore, expert judgment was used to evaluate the validity/credibility of these studies.

Many laboratory studies were performed using static or semi-static exposure regimes, and the reported effect concentrations were frequently based on nominal concentrations, due to a lack of analytical verification of the test concentrations over the study duration. TBT is adsorptive and test concentrations in non-flow-through studies are likely to be highly variable. This also means that equipment can easily be contaminated, thus affecting the actual exposure concentrations. In addition, a variety of units have been used to express the TBT concentration in the literature. To aid comparisons between studies, concentrations are expressed hereafter in a common unit (TBT ion). However, for simplicity and since the difference in molecular weight is small, any concentrations reported as TBTO (bis(tributyltin) oxide) or TBTCl (tributyltin chloride) were assumed to be effectively the same as TBT. Conversion to TBT was therefore made when the unit was originally expressed in terms of tin (Sn) and was accomplished by applying a factor of 2.44, which is the difference in molecular weight.

Other important limitations of the data included wide spacing between test concentrations and consequently between the LOEC and NOEC in some laboratory studies (very few report data in terms of EC_x values), poor methodological descriptions/statistical analyses, and uncertainties in the association between reported dissolved concentrations and observed effects in field studies (since the concentration at the time of adverse event initiation may be different, and organisms may accumulate TBT over a long period).

3 Environmental Fate and Occurrence

3.1 Physical and Chemical Properties

Several tributyltin compounds exist, but in general they all rapidly dissociate in water to form the tributyltin (TBT) cation, which is the toxic moiety. An example is bis(tributyltin) oxide ("tributyltin oxide" or TBTO), which has a vapor pressure of less than 0.016 Pa at 20 °C, a water solubility in the range 0.7–71 mg/L at 20 °C

(depending on pH), and an octanol-water partition coefficient (K_{ow}) around 3.5 (as a log value) (e.g., ECHA 2008; EU 2005; US EPA 2003). The pH-specific K_{ow} (D_{ow}) for TBT is constant above pH 6 (Arnold et al. 1997). An organic carbon-water partition coefficient of 32,000 ($\log_{10} K_{oc} = 4.5$) was proposed by Meador (2000).

3.2 Uses

Historically, TBT compounds were widely used as biocides in antifouling products, parasite control products, and wood preservatives. Regulatory controls have been implemented in many jurisdictions (e.g., under the International Maritime Organization's Anti-Fouling Systems Convention), but some residual biocidal uses in these and other types of industrial and consumer products (including sports clothing) may still occur in some parts of the world (Antizar-Ladislao 2008; Choudhury 2014). They are also used as chemical intermediates for the production of other organotins (e.g., dibutyltin stabilizers for PVC), and may therefore occur as unintentional impurities (e.g., OECD 2007). However, these uses are declining.

3.3 Metabolism

TBT can be metabolized sequentially by cytochrome P450 (CYP450) enzymes to dibutyl- and monobutyltin (e.g., Cooke et al. 2008; Strand et al. 2009). Metabolic capabilities vary widely among taxa (e.g., Ohhira et al. 2003, 2006a, b; Bartlett et al. 2007; Oehlmann et al. 2007; Yang et al. 2009) and even between sexes in mammals (Ohhira et al. 2006b). High bioaccumulation levels in invertebrates and fish are believed to be due to a low capacity for metabolism/excretion and high rates of uptake (Meador 1997).

3.4 Potential Exposure Routes

TBT has a degradation half-life of days to months in water and up to several years in sediment (e.g., ECHA 2008). It is very bioaccumulative, with whole fish bioconcentration factors in the range 2000–50,000 L/kg. The bioaccumulation potential in molluscs can be higher, but is generally similar to that for fish (e.g., Meador 2006; ECHA 2008). TBT bioaccumulation does not follow equilibrium partitioning (Meador 2000). Consequently, some fish exhibit high bioaccumulation factors, which may result from high rates of ventilation. Aquatic organisms can be exposed via both the water column and ingestion of contaminated food (including sediment), although there are no data to suggest that biomagnification occurs in

food webs. Terrestrial organisms may also be exposed via TBT-contaminated sediments (e.g., during flood events or disposal of sediment dredging), application of biocidal products and/or contaminated sewage sludge to soil, atmospheric deposition, and by ingestion of contaminated food or water (Antizar-Ladislao 2008; Silva et al. 2014).

3.5 *Environmental Concentrations*

For this review we did not conduct an exhaustive search for environmental concentrations, but examined past compilations. Several review articles describe the occurrence of butyltins in water from locations around the world over the past several decades (Fent 1996; Antizar-Ladislao 2008). More recent monitoring performed in English and Welsh estuarine surface waters during 2012–2014 was selected as representative of current aquatic levels in a region known to be previously contaminated (UK Environment Agency, pers. com. 2016). The dataset has a number of non-detects; however, the following information is considered to be reasonably conservative for aqueous concentrations: 90th percentile: 0.5 ng/L; arithmetic mean: 0.3 ng/L; median: 0.2 ng/L; range: 0.1–8 ng/L ($n = 269$; two outlier values, 44 and 1368 μg/L, were removed prior to the calculation).

A recent review of tissue concentrations for biota from a variety of countries indicated relatively high TBT concentrations in molluscs, fish, aquatic birds, and marine mammals ranging from low ng/g to low μg/g concentrations, although there were indications of significant decreases with time in some species (Elliott et al. 2007; Mizukawa et al. 2009; Meador 2011).

4 Primary Molecular Initiating Event (MIE)

4.1 *In Vitro and In Silico Analyses*

Binding to a member of the nuclear receptor superfamily is a common MIE associated with endocrine and metabolic pathways. All relevant in vitro assays currently utilize mammalian receptors, assuming interspecies conservation of the structural and functional aspects of each receptor. Among those methods, ToxCast™ uses high-throughput screening methods and computational toxicology approaches to rank and prioritize chemicals. For the US EPA's Endocrine Disruption Screening Program (EDSP), the initial focus of these screening methods has been on estrogen, androgen, and thyroid (EAT) hormone interactions. Analysis of TBT using ToxCast™ identified activity in ER and AR assays at levels generally in excess of the cytotoxicity limit (Fig. 1). In addition, the EDSP21 Dashboard identified TBT as inactive for EAT screens (http://actor.epa.gov/edsp21/).

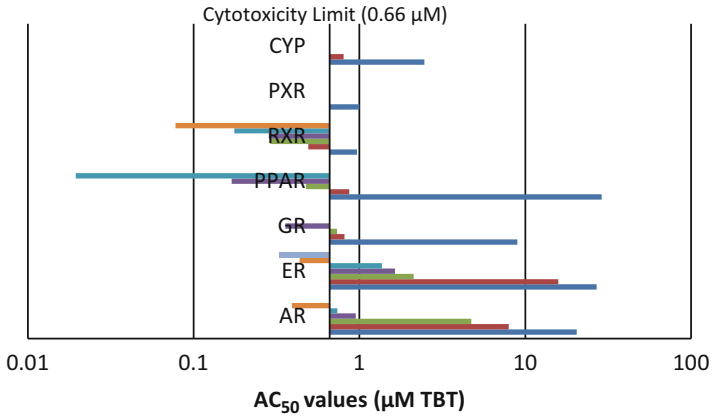


Fig. 1 Selected ToxCast™ nuclear receptor family AC_{50} data for TBT (downloaded March 2016). *CYP* Cytochrome P450, *PXR* pregnane X receptor, *RXR* retinoic X receptor, *PPAR* peroxisome proliferator-activation receptor, *GR* glucocorticoid receptor, *ER* estrogen receptor, *AR* androgen receptor

However, TBT activity in RXR and PPAR assays typically occurred at levels less than the cytotoxicity limit and at lower levels than most ER or AR assays. Thus, ToxCast™ identified the potential of TBT to act through activation of RXR and/or PPAR pathways, although TBT may have been missed as a potential EDC if screening was limited to EAT-related assays alone.

Using an RXR-permissive PPARγ reporter cell line, Grimaldi et al. (2015) demonstrated that, among other butyltins, TBT is able to activate RXR at nanomolar concentrations. Structural profiling using in silico 3D-modelling of the ligand-binding pocket (LBP) of the mollusc *Lymnaea stagnalis* RXR showed that amino-acid residues involved in the binding of RXR ligands (organotins, including TBT, and cis-9-retinoic acid) are identical between *Lymnaea* and humans (Boulahtouf et al. 2015). In addition, the RXR receptor from the freshwater mollusc *Biomphalaria glabrata* does not only bind retinoic acid, but also activates transcription (Bouton et al. 2005). Other studies on invertebrate RXRs have identified significant differences in the LBP as well as a number of mutations that result in low or no affinity of RXR for their vertebrate ligands (all retinoic acids). These include the retinoic acid receptor (RAR) from the marine snail *Nucella lapillus* (Gutierrez-Mazariegos et al. 2014) and the RXR from *Daphnia magna* (Wang et al. 2007), highlighting a knowledge gap regarding the natural ligands relevant to RXR signaling pathways in invertebrate species. We anticipate that with the increasing genomic resources for these important phyla, both the structure and the function of nuclear receptors such as RXR can be further elucidated. This will aid not only our understanding of evolutionally processes but also the risk posed by chemicals such as TBT, which can activate these important transcription factors.

4.2 Weight-of-Evidence for RXR and PPAR$_\gamma$ Pathways

4.2.1 Molluscs

From both in vivo (including injection experiments) and in vitro (transactivation assays) studies, there is strong evidence that TBT interacts with RXR in both marine (e.g., Castro et al. 2007) and freshwater (Boulahtouf et al. 2015) gastropod species. Such interactions were observed at TBT concentrations of 1 nM TBT (equiv. 290 ng TBT/L) in transactivation assays and 1000 ng TBT/g body wet weight through injection. It has also been shown that RXR gene transcription is modulated by TBT, with an increase in the penis-forming area of imposexed females (Lima et al. 2011). Hence, interaction of TBT with RXR seems to be the main molecular event initiating changes in the development of sexual organs in female snails, ultimately resulting in imposex. Importantly, imposex can be induced by cis-9-RA, the natural ligand of RXR (Nishikawa et al. 2004; Castro et al. 2007). More recently, the use of an open transcriptomic approach supported the involvement of steroid, neuroendocrine peptide hormone dysfunction and retinoid mechanisms in imposex induction by chronic exposure to TBT in *Nucella lapillus* (Pascoal et al. 2013). This study also suggested the involvement of PPAR pathways and showed that rosiglitazone, a well-known vertebrate PPAR$_\gamma$ ligand, induced imposex. Nevertheless, although it is certain that activation of RXR and/or RXR/PPAR is the MIE, the mechanistic links with subsequent pathways remain largely unexplained.

However, other primary molecular mechanisms for TBT effects have also been proposed. These include the activation of gonadotropin releasing hormone receptor (GnRHR) along with the gonadotropin releasing hormone (GnRH) (Castro et al. 2007). In *Octopus vulgaris*, it has been shown that a GnRH-like peptide contributed in vitro to an elevation of basal steroidogenesis of testosterone, progesterone, and 17β-oestradiol, in a concentration-dependent manner in both follicle and spermatozoa (Kanda et al. 2006). Another hypothesis formed around the imposex phenomenon suggested that TBT is a neurotoxicant, causing the aberrant secretion of neurohormones, primarily the neuropeptide APGWamide, which regulates male sexual differentiation in molluscs (Oberdorster and McClellan-Green 2002). However, the body of literature is not large enough to allow full evaluation of these additional or alternative pathways.

For many years, the mechanism of TBT-induced imposex was dominated by the steroid hypothesis. This was primarily due to researchers measuring high levels of free testosterone in tissues of impacted molluscs. The link between free testosterone and penis formation was made and supported by numerous publications (e.g., Oehlmann et al. 2007). Despite extensive experimental efforts, treatment with either testosterone or fadrozole (a potent aromatase inhibitor) did not replicate this condition (e.g., Iguchi and Katsu 2008). Although it is well established that TBT does affect many CYPs (including those involved in vertebrate steroidogenesis) and other metabolizing pathways (e.g., esterification), these effects alone do not constitute evidence of a physiological role for these steroids in molluscs. In fact,

both the origin and physiological role of sex steroids in molluscs are still controversial (for reviews see Scott 2012, 2013). Current data suggest that they are likely to be accumulated from the environment. Gooding and Leblanc (2001) provided the first evidence on the ability of molluscan species to take up steroids from water, which was further supported by additional studies (e.g., Janer et al. 2004). More recently, a comprehensive evaluation for steroid uptake by both gastropod snails and bivalve molluscs (Giusti 2013; Giusti et al. 2013a; Schwarz 2015; Schwarz et al. 2017a, b) revealed that this process is very rapid, does not seem to have a saturation limit and is particularly strong for testosterone. Following steroid uptake from the environment, molluscs appear to esterify them (Giusti and Joaquim-Justo 2013) and store them as fatty acid esters through the action of acyl-CoA: testosterone acyl transferase (ATAT) (Janer et al. 2004: Gooding and Leblanc 2001). The retention of steroids as fatty acid esters appears to persist as there is little to no depuration, particularly of estradiol, testosterone, and progesterone from either snails or bivalves placed in clean water for up to 10 days (Schwarz 2015; Schwarz et al. 2017a, b).

Inhibition of either CYPs or phase II metabolism by TBT inevitably will reduce steroid clearance and metabolism, leading to an increase in free steroids. It has been suggested that TBT acts by reducing the retention of testosterone as fatty acid-esters, thus increasing the levels of free testosterone. This may play a role in the development of male sexual organs in females (LeBlanc et al. 2005). However, LeBlanc et al. (2005) added that two assumptions must be met before this putative causative relationship between TBT, testosterone, and imposex can be accepted. First, it must be accepted that testosterone is a male sex-differentiating hormone in molluscs and second, TBT specifically targets some component of the testosterone regulatory machinery causing the aberrant accumulation of this hormone in the snails. None of these has been proven to date. The lack of a nuclear AR or AR-like homologues in the genomes of molluscan species studied to date supports the lack of a physiological role for androgens in these species (Kaur et al. 2015; Vogeler et al. 2014).

4.2.2 Fish

The MIE for reproductive and metabolic impairment in fish is also expected to occur via the RXR and/or PPAR receptors. Lima et al. (2015) exposed zebrafish to only one dose each of TBT, cis-9-RA, and all-trans-retinoic acid in the diet. TBT at this one dose (2.4 μg/g in diet) affected fish weight, fecundity, and sex ratio; however, the natural RXR ligand (cis-9-RA) at 5 μg/g in diet did not. Zhang et al. (2013b) reported significant activation of RXRα in male rockfish (*Sebastiscus marmoratus*) brain when exposed to TBT at 2.4, 24, and 244 ng TBT/L. Female rockfish exhibited the opposite pattern with significant reductions in expression at the two highest doses. No differences in expression of $PPAR_{\gamma}$ were found for males or females at any dose. Several studies reported TBT-induced effects on adipogenesis via RXR/PPAR activation (Meador et al. 2011; Tingaud-Sequeira et al. 2011; Ouadah-Boussouf and Babin 2016).

How exactly the RXR/PPAR activation affects reproduction in fish however is poorly understood. Diverse evidence point to the existence of RXR/PPAR and ER cross-talk. It has been shown however that PPAR and RXR, or their heterodimer, can bind directly to estrogen responsive elements (EREs) in the gene promoters (Nunez et al. 1997). Other studies suggest that RXR/PPAR activation directly affects CYP expression, including aromatase (Cheshenko et al. 2008). A PPAR/RXR responsive element was predicted in the zebrafish CYP19β promoter (Kazeto et al. 2001) whilst a RAR binding region was identified in the tilapia CYP19 promoter (Chang et al. 2005). This was further supported by in vitro studies with mammalian cell lines where TBT binding to RXR and PPAR leads to modulations of CYP19 expression (Nakanishi et al. 2005, 2006).

4.2.3 Mammals

As described above, Kanayama et al. (2005) presented some of the first evidence of the implication of the RXRα and $PPAR_{\Upsilon}$ pathways in TBT-mediated endocrine effects in mammals, and also proposed that this pathway is the likely route for the low-dose imposex response in gastropods. Additional studies (Nakayama et al. 2005; Grün et al. 2006; Le Maire et al. 2009) provided further data showing that TBT exerts its biological effects via transcriptional regulation of gene expression through activation of these receptors, implicating this pathway in the adipogenic effects of TBT. Mammalian aromatase gene expression is also regulated through the RXRα/$PPAR_{\Upsilon}$ pathway by various ligands, including TBT. However, the direction of the regulation in response to TBT appears to be dose- and tissue-specific, potentially because aromatase is regulated through tissue-specific promoters (Simpson et al. 1993).

4.2.4 Summary

Retinoic acid binds to both RAR and RXR regulating the transcription of 500 genes involved in a large array of biological processes (Blomhoff and Blomhoff 2006). RA has long been recognized as a morphogen, important for axial patterning and organ formation in developing vertebrates. An adverse outcome pathway for neural tube and axial defects modulated by retinoic acid in vertebrates (including man) has been proposed (Tonk et al. 2015). This analysis was based on data from rat whole embryo culture, embryonic stem cells and the zebrafish embryotoxicity test, and identified certain conserved pathways on RA signaling between mammals and fish. Adverse effects in vertebrates included craniofacial and limb malformations/defects, which suggests that the TBT-induced developmental abnormalities in mollusks, such as shell abnormalities (Alzieu et al. 1986), and imposex are different expressions of disruption of the same pathway. Analysis of genomic data revealed that the important morphogenic role of RA does not only extend to invertebrate

chordates (tunicates and cephalochordates), but also to other invertebrate groups, such as hemichordates and sea urchins (Campo-Paysaa et al. 2008).

Altogether evidence suggests that most of the endocrine effects of TBT have their origin in RXR and RXR/PPAR activation. An important determinant of the severity and magnitude of responses in different species is the structure of the ligand binding domain, as studies have demonstrated the evolutionary plasticity of this domain, whilst the function of RAR, RXR, and PPAR appears to be largely conserved.

5 Toxic Effects Plausibly Mediated by Endocrine Disruption

5.1 The OECD Conceptual Framework for Endocrine Disruptor Testing and Assessment

As previously mentioned, few studies were performed according to standard test guidelines that correspond to the OECD Conceptual Framework for Endocrine Disruptor Testing and Assessment (CFEDTA; see Table 1 of the Annexes) (OECD 2012a, b). Non-standard studies do not necessarily fit within the different levels of this framework. Hereafter, the studies with TBT are therefore organized according to the test design, including the exposure duration and portion of the life-cycle exposed, and to the type of biological responses. As EC_x were not always provided or could not be deduced from the study, endpoints are mainly reported as LOEC and/or NOEC.

Standard ecotoxicology data characterizing acute toxicity endpoints were not reviewed in any great detail for this review because overt toxicity did not impact the evaluation of endpoints relevant to endocrine disruption. Acute toxicity for sensitive species was generally one to two orders of magnitude greater than LOECs for the relevant chronic apical and endocrine disruption endpoints.

5.2 Non-test Approach: Summary of Toxicological Information

The level 1 of the OECD CFEDTA corresponds to non-test information that can be used to define the general toxicological profile of a chemical with respect to its endocrine disruption properties (OECD 2012a). QSAR analyses have been conducted in some organisms (e.g., green algae; Neuwoehner et al. 2008). However, they only provide patchy information and do not appropriately cover taxa of interest such as molluscs or fish. Therefore, in the context of the present review, TBT has been evaluated in the US EPA's ToxCast™ program (http://epa.gov/ncct/toxcast/). It was classified as being a "promiscuous" chemical because it exhibited

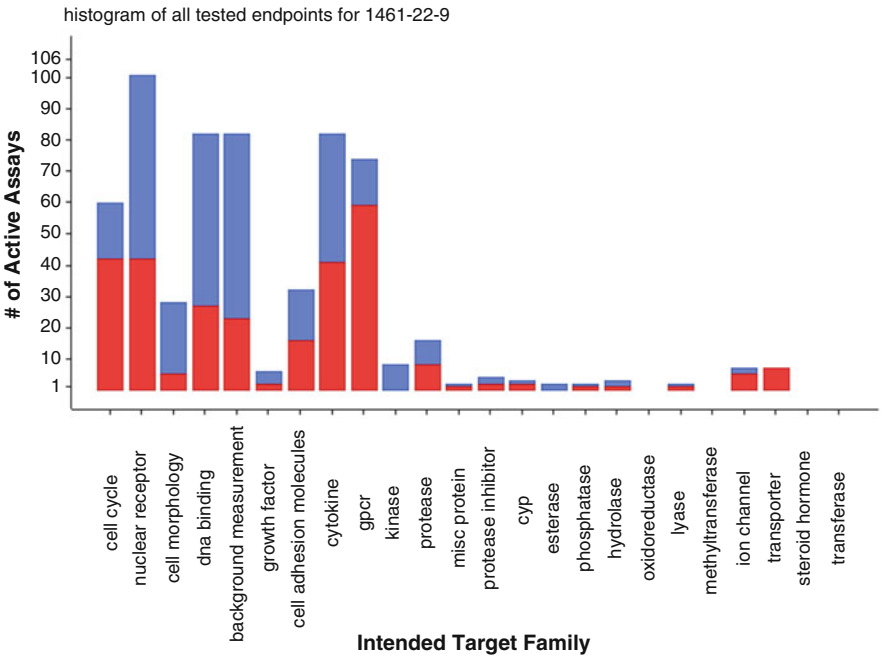


Fig. 2 Result of the ToxCast™ analysis for TBT (*red* = active assays and *blue* = inactive assays) (Downloaded March 2016)

activity in approximately 285 assays across 20 target families (Fig. 2). This type of profile suggests that TBT will act across a number of toxic pathways potentially including endocrine and non-endocrine molecular initiating events.

5.3 Invertebrates

5.3.1 In Vitro Assays

In molluscs, few studies have reported the use of in vitro assays for identifying initial endocrine mechanisms (i.e., MIE) due to TBT exposure. These studies employed transactivation assays using mammalian cells transfected with RXR from *Thais clavigera* (Urishitani et al. 2013) and *Lymnaea stagnalis* (Boulahtouf et al. 2015). The results demonstrated that TBT binds the LBD of RXR in these species, and its affinity is equivalent to that of 9-cis-RA, the natural ligand of RXR.

5.3.2 Physiological Responses

In the reproductive tissues of female dog whelk *N. lapillus*, RXR gene transcription is increased in the penis-forming area associated with the formation of a penis

and/or vas deferens (Lima et al. 2011). In this species, 9-cis-RA is as potent as TBT in inducing imposex, indicating that TBT toxicity in gastropods is mediated through the modulation of the RXR signaling pathways (Nishikawa et al. 2004; Castro et al. 2007). It should be noted that imposex is also induced by rosiglitazone, a PPARγ agonist, suggesting that the heterodimer of RXR-PPAR is a critical pathway for this phenomenon, at least in *N. lapillus* (Pascoal et al. 2013). This study also suggested that other transcription factors such as LXR and lipophilic orphan receptors are involved in the TBT toxicity pathway.

Interestingly, TBT appears to affect the endocrine system of insects as well (Hahn and Schulz 2002). In an in vivo study on *Chironomus riparius*, an environmentally relevant concentration of TBT (1 ng/L) showed effects on many endocrine-related genes, including up-regulation of the ecdysone receptor, the ultraspiracle gene (the orthologue of RXR in insects), the estrogen-related receptor, and the E74 early ecdysone inducible gene, whilst the vitellogenin (Vtg) gene remained unaffected (Morales et al. 2013). The same study showed genotoxic effects of TBT in insects by means of the comet assay.

5.3.3 Organismal Effects

A wide variety of organ responses are known in molluscs exposed to low concentrations of TBT. These include penis development in female snails, abnormal testis histopathology, and sperm alteration (count, motility, abnormality). These responses occur in the 1–10 ng TBT/L range for aqueous concentrations in the environment (e.g., Horiguchi et al. 1994; Leung et al. 2006) and 10 to 100 ng TBT/g wet weight (ww) whole-body tissue (Meador 2011) in affected gastropods. Female prosobranch molluscs exposed in the laboratory can develop imposex with LOECs of 1 to 83 ng/L (Gooding et al. 2003; Abidli et al. 2012, 2013).

In gastropods, adverse effects resulting from in vivo exposure to TBT mainly concern reproductive impairment associated with alterations of the sexual organs in females, including the staged development of penis and vas deferens known as imposex. The Vas Deferens Sequence Index (VDSI) has been developed to characterize the extent of imposex in *N. lapillus* and *T. clavigera* (e.g., Gibbs et al. 1987; Blackmore 2000; Leung et al. 2006). VDSI has seven stages, with stage 0 indicating no imposex and stage 6 indicating female sterilization. Several studies indicate that the imposex threshold concentration lies at 1 ng TBT/L, with increasing sterilization as concentrations increase (e.g., Gibbs et al. 1988). Gibbs (1996) found that juvenile female *Ocenebra erinacea* exposed to 7.3 ng TBT/L developed a longitudinal split of the oviduct wall. Adult *O. erinacea* collected from TBT-contaminated sites with advanced imposex exhibited the same lesions and through laboratory spawning experiments were found to be sterile (no capsules produced). Significant reductions in gastropod reproduction were generally found at levels slightly higher than those that induced imposex, with effective concentrations ranging from 12 to 1000 ng TBT/L (Duft et al. 2007; Leung et al. 2007; Giusti et al. 2013a, b). Other invertebrate species are known to exhibit reproductive effects across a wide

aqueous concentration range of 10 to 2225 ng TBT/L (Oberdorster et al. 1998; Ohji et al. 2003a, b; Huang et al. 2010).

Developmental toxicity of TBT was also investigated, using a 21-day embryo test with the freshwater snail *Lymnaea stagnalis*. TBT (added as TBTCl) had NOEC values of 30 ng TBT/L and 100 ng TBT/L for the mean hatching time and hatching success, respectively (Bandow and Weltje 2012). These values compare well with the NOEC of 105 ng TBT/L obtained for the fecundity of adult *L. stagnalis* exposed to TBT hydride for 21 days (Giusti et al. 2013a, b).

5.3.4 Life-Cycle Studies and Population-Level Responses

Limited full life-cycle or multigenerational laboratory studies using TBT have been conducted with invertebrates other than molluscs. The calanoid copepods *Pseudodiaptomus marinus* and *Schmackeria poplesia* exhibited similar sensitivities, with LOECs of 6 and 20 ng TBT/L, respectively (Huang et al. 2006, 2010). The amphipod *Caprella danilevskii* exhibited similar sensitivity to the copepods with a LOEC of 10 ng TBT/L (Ohji et al. 2003a, b). *Daphnia magna* was thought to be much less sensitive to TBT, with a LOEC of 2225 ng TBT/L in a two-generation study (Oberdorster et al. 1998). However, a recent study reported reduced brood size, total offspring, neonate volume, and neonate length at 88 ng TBT/L, with many of these effects being observed in the F1 and F2 generations (Jordão et al. 2015). Altered lipid homeostasis was suggested to be responsible for the abnormalities in reproduction.

For gastropods exposed to TBT, life-cycle and population studies are mainly represented by long-term laboratory exposures (>1 year) or field monitoring. In gastropods, a large number of studies reported decline to extinction for populations with increasing proportions of imposexed females (Gibbs 1996, 2009). Several studies indicate that TBT in the marine environment can impact populations of Caenogastropod snails through female sterilization associated with imposex (Spence et al. 1990; Bailey et al. 1995; Harding et al. 1997). These population-level responses were associated with water concentrations in the range 1–10 ng TBT/L, which is consistent with molecular studies characterizing the affinity of TBT for the RXR-PPAR receptor, in addition to other known ligands. It is important to note that there is not always a linear relationship between imposex development, as assessed through the VDSI, and female sterility (Barroso et al. 2002). On the other hand, female gametogenic activity occurs with no apparent differences in imposex-affected and imposex-free populations (Avaca et al. 2015). This may have important implications for population recovery.

Shell development in molluscs may be as sensitive as imposex and reproduction with a number of species exhibiting shell growth and development effects in aqueous concentrations ranging from 8 to 1000 ng TBT/L (Leung et al. 2006, 2007; Giusti et al. 2013b). Shell abnormalities in oysters were reported as early as the beginning of the 1980s (Alzieu et al. 1986), showing that oyster populations have also been impacted by TBT. For example, a correlation was found between

rock oyster (*Saccostrea glomerata*) population density and the discontinued use of TBT in estuaries with high densities of boat moorage (Birch et al. 2014). This conclusion is also supported by other studies that reported declining tissue concentrations in molluscs from this area over the same time period (Batley et al. 1992; Lewis et al. 2010).

5.3.5 Reversibility of the Effects

The degree of imposex reversibility in molluscs depends on the species. Once TBT exposure stops, female penis length declines slowly in *Nassarius reticulatus* (Bryan et al. 1993), and more quickly in female *Ilyanassa obsoleta* (Smith 1981). Contrary to this, imposex in *N. lapillus* is largely irreversible (Bryan et al. 1987). Population recovery can therefore be slow, especially for species that are long-lived and/or for which recruitment is limited, such as *N. lapillus* (Matthiessen and Gibbs 1998; Oehlmann et al. 2007; OECD 2010) and *N. reticulatus* (Couceiro et al. 2009). Nevertheless, there appears to be a widespread amelioration worldwide (e.g., Canada, US EPA 2003; Hong-Kong, Leung et al. 2006; Spain, Couceiro et al. 2009; England and Wales, Nicolaus and Barry 2015) as populations of snails have recovered significantly after the reduction in use of TBT as an antifoulant (Birchenough et al. 2002; Bray et al. 2012; Nicolaus and Barry 2015).

5.4 Fish

5.4.1 In Vitro Assays

Organotins, including TBT, are well-known inhibitors of the hepatic microsomal CYP450 systems in a variety of fish species (review by Fent and Hunn 1996). Protein transcription, enzyme activity, and reductases are all affected. The isoform CYP450 1A1 appears to be particularly sensitive to TBT exposure. Other CYP450 isoforms, including those with testosterone hydroxylase activity, are also inhibited, albeit at high concentrations. In general, CYP450 inhibition occurs at levels that are close to cytotoxicity and as such it is difficult to establish whether the inhibitions observed constitute an additional mechanism of toxicity, or they are indeed an effect of general toxicity affecting a variety of sulfhydryl-containing proteins. It has since been established that not only phase I but also phase II metabolism enzymes are affected by TBT (Morcillo et al. 2004). It is interesting to note that in this study, glucuronidation of testosterone but not estradiol was inhibited by incubating fish liver microsomes with TBT at concentrations as low as 5 μM.

The general inhibitory effect of moderate to high doses of TBT on hormonal and biotransformation pathways has also been confirmed in salmon hepatocytes using gene expression patterns (Vtg, ER, AR) and CYP-mediated enzyme activities as endpoints (Mortensen and Arukwe 2009). These consistent decreases in cellular

responses over time and with increasing TBT concentrations suggest a possible inhibitory effect of TBT on transcription. The effect of TBT on other transcription factors such as RXR and PPAR in fish cell systems is surprisingly under-studied. Nevertheless, TBT inhibited plaice (*Pleuronectes platessa*) $PPAR_{\alpha}$ and $PPAR_{\beta}$ at 1 nM in transfection assays although it had no effect on $PPAR_{\gamma}$ (Colliar et al. 2011).

5.4.2 Physiological Responses

Several studies have examined various physiological responses in fish exposed to TBT. A series of studies have been published on TBT-induced effects on rockfish (*Sebastiscus marmoratus*) gonadal development. In the first study, Zhang et al. (2007) brought wild female fish into captivity for experimentation. Ovarian testosterone levels of fish exposed to 10 ng TBT/L significantly increased, whereas levels in ovaries exposed to 100 ng/L did not significantly change compared to the control. Exposure to 10, 100 ng/L TBT resulted in significantly decreased 17β-estradiol levels in the ovaries. No masculinization was observed but the exposure period, limited to 50 days, might have been too short or the sexual differentiation stage as female was too advanced. Histological examination reportedly showed developmental suppression and atresia of ovarian follicles. However, these results may be unreliable as they included incorrect criteria to judge the follicles development stage [e.g., follicles labeled vitellogenic were not vitellogenic, and the results of Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay staining for apoptosis were not convincing; J. Wolf, pers. com. 2016]. Likewise, there was no mention of methods used to minimize observational histopathological bias.

In a second experiment (Zhang et al. 2009a, b), fish were exposed to nominal concentrations of 2.44, 24.4, and 244 ng TBT/L for 48 days. There was strong evidence of inhibition of testicular development, interstitial fibrosis and increased testicular lipid but no NOEC (only a LOEC) was established. Histopathological testicular changes were observed that may represent a stress response as opposed to specific endocrine or toxicological activity (Zhang et al. 2009a). Inhibition of thyroidal status related to depression of testicular development was also studied at TBT concentrations of 2.44, 24.4, and 244 ng TBT/L for 50 days. The NOEC was 2.44 ng TBT/L based on histopathology (testis: decreased spermatozoa, pyknosis, interstitial fibrosis; thyroid: decreased colloid). There was a possible effect of decreased spermatozoa at 244 ng TBT/L, and damage to the thyroid gland and a decrease in production of thyroid hormones were observed (T4 in serum significantly correlated with GSI) (Zhang et al. 2009b).

In a third study (Zhang et al. 2011), TBT-induced RXR_{α} expression in embryos at 0.1 and 1 ng/L, an effect thought to be responsible for the induction of increasing apoptotic cells. TBT was also shown to induce ectopic lipid accumulation in ovarian interstitial cells (consistent with PPAR activation) and decreased testosterone esterification in the ovaries leading to increased free testosterone (NOEC = 1 ng/L).

In a final study (Zhang et al. 2013a, b), 30 male and 30 female fish per group were exposed to nominal concentrations of TBT of 1, 10, and 100 ng/L. CYP19b expression in the male fish significantly increased ($p = 0.026$, 0.04, and 0.02, respectively) after exposure to TBT, the highest elevation being 3.12-fold in the 10 ng/L group. In the female fish, the CYP19b expression increased slightly in the 10 and 100 ng/L groups, but this was not statistically significant ($p = 0.078$ and 0.234, respectively). Testosterone and estradiol levels were unchanged in males but testosterone increased and estradiol decreased in females. ER expression was affected in males (up-regulated at low concentrations; no difference at 100 ng/L) but not in females. RXR_{α} expression increased in males but decreased in females.

TBT is a complex endocrine disrupter in zebrafish (*Danio rerio*). McGinnis and Crivello (2011) injected TBT at 1–5 mg/kg intraperitoneally into fish, which directly inhibited ER-regulated processes by acting as a non-competitive inhibitor. TBT did not inhibit AR-regulated processes, but decreased acyl-transferases and sulfation of testosterone in the liver. TBT had an androgenizing effect in the brain but a feminizing effect in the liver and gonads. Rapid metabolization of TBT to di- and monobutyltin also occurred in the liver, resulting in complex and non-elucidated interactions with steroid pathways (McGinnis et al. 2012).

In the brown trout (*Salmo trutta fario*), TBT exposure showed a decreasing trend of ovarian CYP19 expression, but not a potent effect at 1000 ng/L (Pereira et al. 2011a, b), suggesting that TBT interferes with the steroidogenic pathway at a transcriptional level. The in vivo down-regulation of IGF2 in the pre-vitellogenic ovaries might indicate that TBT interferes with factors that are absent in the ex vivo gonad cultures (Pereira et al. 2011a, b). TBT did not affect testosterone or estradiol concentrations, further supporting previous evidence that the CYP19 modulating effects of this chemical are not mediated through direct inhibition of CYP19 activity (Pereira et al. 2011a, b). Aromatase expression in the brain, reproductive behavior, and secondary sexual characteristics were studied by Tian et al. (2015) in guppies (*Poecilia reticulata*). TBTCl treatment inhibited gene expression of CYP19A and CYP19B in brain of males, which led to altered reproductive behavior with a LOEC of 4.45 ng TBT/L.

A series of studies with juvenile Atlantic salmon (*Salmo salar*) that were force-fed TBT alone and in combination with forskolin, reported a number of affected gene expression patterns, including CYP3a, CYP11b, CYP19a, SF-1, glucocorticoid receptor, ERα PXR, PPARs, glutathione *S*-transferase (GST), ACOX 1, IL-b, TNFa, IFNγ, IFNα, Mx3, IGF-1, IL-10, and TGFb (Kortner et al. 2010; Pavlikova et al. 2010, Pavlikova and Arukwe 2011). Forskolin activates the enzyme adenylyl cyclase and increases intracellular levels of cAMP, an important second messenger necessary for the proper biological response of cells to hormones and other extracellular signals. Since most effects observed after TBT exposure were modulated by forskolin exposure, these studies suggested that TBT may exert its endocrine, biotransformation and lipid peroxidation effects via the cAMP/PKA second messenger system.

5.4.3 Organismal Effects

In contrast to the extensive literature dealing with the adverse impacts of TBT in mammalian and molluscan species, relatively few studies have addressed higher-level effects of TBT on fish. Bentivegna and Piatkowski (1998) studied embryotoxicity of TBT in medaka *Oryzias latipes* exposed to nominal concentrations of TBT acetate (TBTA). Results showed that 415 and 4150 ng/L produced 100% lethality in all age groups, while 41.5 ng/L produced no acute lethality in 3- and 5-day embryos, and between 16 and 33% lethality in 0-day embryos. Subchronic endpoints showed that toxicity was concentration-related and that embryos exposed on day 0 were more sensitive than those exposed on days 3 and 5. LOECs for hatching success were 10,440 ng/L for day 0 and 41,500 ng/L for days 3 and 5. LOECs for the combined effects of hatching success and gross abnormalities were 10,440 ng/L for day 0 and 20,590 ng/L for days 3 and 5. Although no endocrine-sensitive endpoints were measured, there was some evidence of reduced CYP450 induction.

It has been reported that TBT can alter the sex ratio towards males in zebrafish (McAllister and Kime 2003; Santos et al. 2006) and Japanese flounder (*Paralichthys olivaceus*) (Shimasaki et al. 2003). Genetic female flounder exhibited an increased rate of sex reversal when fed TBT in their diet. The proportion of males significantly increased to 25.7% in the 0.1 μg/g group and to 31.1% in the 1.0 μg/g group compared with the control (2.2%). Histological observations showed that, in the TBT-treated groups, normal females had typical ovaries and assumed sex-reversed males had typical testes without intersex (Shimasaki et al. 2003). Zebrafish exposed from 0 to 70 days post-fertilization (dpf) to 0.1 ng/L of TBT showed a male-biased population and produced a high incidence of sperm lacking flagella. At 1 ng/L, the motility of sperm was significantly lower than that of control fish, while at 10 ng/L, all sperm lacked flagella, and at 100 ng/L, milt volume increased. Male sex ratio shifts were similar after exposure from 0 to 70 dpf and 0 to 30 dpf. Equally important, 100 ng/L resulted in 65% males after exposure from 30 to 60 days emphasizing the point that timing of the exposure is very important. Effects on sperm motility and morphology and on milt volume were less pronounced after 30 days than after 70 days of exposure (McAllister and Kime 2003). From this study, the NOEC and LOEC values were 0.01 and 0.1 ng/L, based on nominal exposure concentrations. In a recent study, Lima et al. (2015) exposed zebrafish larvae from 5 dpf up to 120 dpf to 1466 ng TBT/g diet. Animals were fed this diet three times per day. Females were significantly smaller and weighed less, while no change in male total length or weight was observed. Gonad weight in males was significantly heavier but no change was observed in females. There was a 62% decrease in fecundity but no changes in egg viability or hatchability. Overall, sex ratios shifted towards females in contrast to other studies reported above. This could be a strain difference or a concentration effect. The expression of gonadal aromatase was unaffected but in female brain, TBT down-regulated CYP19a1b mRNA. There was also a brain-specific down-regulation of $PPAR_{\gamma}$ in both males and females. TBT effects in zebrafish may involve

modulation of $PPAR_{\gamma}$/RXR and brain aromatase based on this study. A LOEC for all endpoints of 1466 ng TBT/g diet was reported, based on measured concentrations.

Reduced sperm counts were observed in guppies (*P. reticulata*) following exposure to 11.2 or 22.3 ng TBT/L for 21 days, with possible effect on Sertoli cell function. This potentially occurred via apoptosis, which could block the nutritional activity of Sertoli cells on maturing spermatids and thereby arrest the release of gametes. TBT exposure for 21 days decreased sperm counts in guppies by 40–75% but flagellar length was unaffected. However, this exposure involved adult fish and effects on sperm were short-term. Sperm counts declined approximately 62–69% but there was no change in testes size or sperm length (Haubruge et al. 2000). In a histological evaluation of TBT's toxicity on spermatogenesis, Mochida et al. (2007) exposed mummichog (*Fundulus heteroclitus*) to mean measured TBT concentrations of 0 (control and solvent control), 0.20, 0.54, 1.0, 1.7, 1.9, and 2.8 μg/L. In this study, there was a relatively small group size and no mention of the methods used to minimize sampling or observational bias. Some of the histopathological changes were difficult to confirm at low magnification, and there were several low quality figure images (changes could also be autolysis). However, damage to epithelial cells of seminal ducts, and slight decrease in spermatozoa numbers were reported with a NOEC-based on histopathology of 1.7 μg TBT/L. TBT can also affect sexual behavior and reproduction in medaka (*Oryzias latipes*) (Nakayama et al. 2004).

Growth effects in fish were also found at very low tissue concentrations. Shimasaki et al. (2003) reported a statistically significant decrease in body weight and length at 18 ng/g body wet weight; however, Meador et al. (2011) found significant increases at essentially the same whole-body concentration in Chinook salmon. Increased growth and lipid content data reported by Meador et al. (2011) are consistent with the mammalian response data characterizing TBT as an obesogen (Grün et al. 2006).

Several studies examined effects of maternally transferred TBT using different routes of exposure including dietary (Nakayama et al. 2005; Shimasaki et al. 2006) and injection (Hano et al. 2007). Adverse effects were noted when TBT concentrations were approximately 5–160 ng/g egg wet weight. These studies support the conclusion that fish embryos are very sensitive to TBT and indicate that maternal transfer is an important route of exposure.

5.4.4 Life-Cycle Studies

Mochida et al. (2010) exposed mummichog *Fundulus heteroclitus* in a fish full-life-cycle assay from the embryo stage until the hatch of the F1 generation at nominal TBTO concentrations of 0 (control and solvent control), 0.13, 0.25, 0.50, and 1.0 μg/L. The mean measured equivalent TBT ion concentrations corresponding to these exposure groups were 0, 0.054 ± 0.005, 0.12 ± 0.02, 0.26 ± 0.02 and 0.37 ± 0.05 μg/L, respectively. In a second experiment, nominal concentrations of

0.13, 0.50 and 2.0 μg TBTO/L were measured as 0.034 ± 0.00, 0.21 ± 0.07 and 0.81 ± 0.02 μg/L, respectively as the equivalent TBT ion. In the F0 generation, TBT exposure resulted in a male-biased sex ratio, an increase in the frequency of the appearance of apoptotic cells in the testis in maturing stages, and a decrease in fecundity. In the F1 generation, time to hatch and hatchability were all markedly affected. Exposure did not affect the proliferation of the germ cells in the testes; however, a significant increase in the number of apoptotic cells in the testes was induced. LOECs for sex differentiation (towards males), reduced spermatogenesis (increased apoptotic cells), and reduced hatching were 0.26, 0.06, and 0.05 μg/L, respectively.

5.5 *Amphibians*

5.5.1 In Vitro Assays

Few studies have been identified that examined the effects of TBT on amphibians in vitro. Using transiently transfected Cos7 cells, Grün et al. (2006) demonstrated that exposure to 60 nM TBT (presumably TBTCl, although compound, purity and source were not specified) activated RXRα and RXRϒ from the amphibian *Xenopus laevis*. Choi et al. (2007) found that TBT inhibited *Rana dybowskii* oocyte maturation in vitro (ED50: 0.6 and 0.7 μM).

5.5.2 Physiological Responses

Mengeling et al. (2016) found that 1 nM TBT (equiv. 290 ng/L) greatly potentiated the effect of T3 on thyroid hormone-induced morphological changes in *X. laevis* but showed both gene and tissue specificity in this capacity. The data also demonstrated that as an RXR agonist, TBT can disrupt TH signaling with outcomes identical to those caused by synthetic RXR-selective ligands and suggested that TBT is not activating a permissive NR-RXR heterodimer such as $PPAR_{\gamma}$ to achieve this effect.

5.5.3 Organismal Effects

Amphibian embryo development and tadpole metamorphosis are also sensitive to TBT exposure. *Xenopus tropicalis* embryos showed developmental and survival effects when exposed to TBT at 50–400 ng/L in the frog embryo teratogenesis assay-*Xenopus* (FETAX) (Guo et al. 2010). These effects were time- and concentration-dependent, with significant mortality at each time interval (24, 36 and 48 h) and exposure concentration. The most common malformations in the embryos were abnormal eyes and skin hypo-pigmentation, with increased time of exposure; additional common malformations included enlarged proctodaeum and

narrow tails fins in tadpoles. Thyroid hormone is linked to eye development in *Xenopus* embryos. The authors suggest that the eye malformations and other malformations are linked to TBT exposure through binding to RXRs and that RXRs form heterodimers with the thyroid hormone receptors.

5.5.4 Life-Cycle Studies

Shi et al. (2014) exposed *X. laevis* to TBT in an Amphibian Metamorphosis Assay (AMA) (OECD TG 231) and a complete AMA (CAMA), which exposed *X. laevis* from Nieuwoop and Faber (NF) stage 46 to stage 66. They found TBT to have anti-thyroid activity in the AMA at TBT concentrations of 12.5–200 ng/L, based on decreased hind limb length in the absence of growth effects (body weight and snout to vent length) or overt toxicity, delayed development by one or two development stages and thyroid lesions characterized by mild increases in thyroid follicle height and/or mild increases in layers of follicular epithelium, and colloid depletion. The CAMA confirmed developmental delays based on front limb emergence and total metamorphosis time; however, these effects were seen in the presence of decreased body length and weight at metamorphosis at 10 and 100 ng/L. The CAMA also found that the intersex and sex ratio increased in favor of males with increasing concentrations of TBT. The intersex gonads had an ovarian cavity with testis-like tissue structure. Apical endpoints in *Xenopus* sp. metamorphosis and embryo eye development were affected by TBT concentrations as low as 12.5 ng/L (Shi et al. 2014). Although these endpoints are regulated by thyroid hormones, it is unclear from this report whether TBT acts directly on thyroid hormones, or indirectly through binding to RXRs.

5.6 Birds

5.6.1 In Vitro Assays

No in vitro studies of TBT effects on birds have been identified.

5.6.2 Physiological Responses and Organismal Effects

Although few studies of the effects of TBT have been conducted in birds, these have demonstrated some reproductive effects from exposure. A subchronic toxicity/reproduction study was performed in Japanese Quail (*Coturnix japonica*) fed a diet containing 0, 24, 60, and 150 mg TBTO per kg diet for 6 weeks (Coenen et al. 1992). No overt toxicity or treatment-related pathological or histological abnormalities were noted in parent birds, and there were no significant effects on egg production, serum alanine aminotransferase (ALAT), serum total thyroxine (TT4),

luteinizing hormone (LH), or retinol levels. Limited effects on hematology and no effects on serum biochemistry were found in these birds (Coenen et al. 1994). However, a significant decrease in hatchability and increase in embryo mortality were observed at the two highest doses. Serum calcium values determined throughout the reproduction period were found to be significantly reduced in female birds at all concentrations tested (Coenen et al. 1992). This same study was repeated by five laboratories in an inter-laboratory comparison test (Schlatterer et al. 1993), with the addition of one higher dose group (375 mg/kg feed). Results from this comparison were similar to those reported by Coenen et al. (1992). Dose-related decreases in egg weight, egg production, fertility, hatching success and survival of 14-day-old chicks were observed in most of the laboratories. The NOEC for egg weight and hatchability was 60 mg TBTO/kg feed. Faqi et al. (1999) conducted a follow-up study to examine whether a difference in responses could be observed between a 6-week exposure and a 13-week exposure to 150 and 375 mg TBTO/kg feed. The number of eggs laid, mean egg weight, fertility and hatchability were significantly lower and the percentage of cracked eggs was significantly higher at 375 mg/kg at weeks 6 and 13. Reduced eggshell thickness was observed at week 6. No effects were noted on hematological and clinical chemistry data obtained at weeks 6 or 13 and histological preparations of the organs showed no morphological changes. However, none of these studies in quail examined steroid hormone levels or any related genomic indicators from the HPG axis, nor were any effects on nuclear receptors investigated.

5.6.3 Life-Cycle Studies

No life-cycle studies of TBT effects have been reported for birds.

5.7 Mammals

5.7.1 In Vitro Assays

Studies using mammalian in vitro systems have examined interactions between TBT and nuclear receptors and the effects of TBT on enzymes involved in steroidogenesis. Saitoh et al. (2001a) found that TBT had relatively high binding affinity for androgen receptor (AR), with an IC50 of 7.6 μM, but no affinity for estrogen receptor α (ERα). Additional evidence for TBT's impacts on segments of the steroidogenic cascade was presented by McVey and Cooke (2003), who observed decreased 17-hydroxylase and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in rat testis microsomes at 12 and 59.0 μM TBTCl. Treatment of human granulosa-like tumor cells for more than 48 h with 20 ng/mL TBTCl (~60 nM), significantly suppressed aromatase activity and estradiol production (Saitoh et al. 2001b). Cooke (2002) and Heidrich et al. (2001) reported that TBT

is a competitive inhibitor of human aromatase in vitro at 12 and 59 μM TBTCl and 5 and 50 μM TBTCl, respectively, with an IC50 of 6 μM calculated by Heidrich et al. (2001). Together, these studies initially suggested a possible inhibitory role for TBT in steroidogenesis as the primary means of endocrine disruption. However, Kanayama et al. (2005) found that TBT (10, 30, and 100 nM TBTCl) induced the transactivation function of RXR_{α} and $PPAR_{\Upsilon}$ at concentrations lower than those causing aromatase inhibition. The effect of TBT on RXR_{α} is as strong as that of its endogenous ligand, 9-cis-RA and because TBT enhanced protein-protein interaction between RXR_{α} and TIF2, the data suggested that TBT activates transcription via these receptors.

Because TBT induced the transactivation function of RXR_{α} and $PPAR_{\Upsilon}$ at concentrations lower than those required for inhibition of aromatase activity, Kanayama et al. (2005) proposed that this receptor-based pathway is the more likely route for low-dose effects such as imposex in gastropods. The greater likelihood of a role for RXR and PPAR over the aromatase-inhibition hypothesis in TBT-mediated toxicity was further supported by studies that reported opposing effects of TBT on aromatase (CYP19). Sharan et al. (2013) found that low doses of TBT (25, 50, and 100 nM TBTCl) increased CYP19 enzyme activity, mRNA expression, and estradiol production in MCF-7 cells and acted as an ERα agonist. Evidence for the induction of aromatase by TBT was initially provided by Nakanishi et al. (2002) at 30, 100, and 300 nM TBTCl in a human choriocarcinoma cell line. Evidence for the role of the RXR homodimer in aromatase induction in placental tissue was provided by Nakanishi et al. (2002, 2005) at 1, 10, and 100 nM TBTCl. However, the utility of these studies for risk assessment is weak due to the use of inappropriate statistical methods. The variation in results of these in vitro studies with regard to aromatase and ER activation is likely due to wide differences in concentrations of TBT and/or the sources for the cells used in each study, suggesting that TBT's effects may be dose- and tissue-specific.

Additional early evidence for interaction of TBT with RXR and PPAR via direct ligand binding was presented by Grün et al. (2006) using Cos7 cells (transformed Green Monkey kidney fibroblast cells) transfected with human, mouse, and frog (*Xenopus laevis*) nuclear receptors. These experiments showed activation of $RXR_{\alpha-\Upsilon}$ and slightly weaker activation of $PPAR_{\Upsilon}$ and $PPAR_{\delta}$ (same for $PPAR_{\beta}$) at 60 nM TBT (presumably TBTCl, although compound, purity and source were not specified). le Maire et al. (2009) showed that TBT activates all three RXR-PPAR heterodimers (α, β and ϒ) primarily through its interaction with RXR. In contrast to the interaction between TBT and RXR, the active receptor conformation between TBT and $PPAR_{\Upsilon}$ was less efficiently stabilized, making this side of the heterodimer a less efficient binding target for TBT (le Maire et al. 2009), confirming the observations of Grün et al. (2006).

Multiple lines of in vitro evidence exist demonstrating the roles of RXR and PPAR in TBT-induced adipogenesis in mammals. Histological examination of mouse 3T3-L1 preadipocyte cells treated with 100 nM TBTCl by Kanayama et al. (2005) revealed induction and promotion of adipocyte differentiation. This observation was supported by induction of the adipocyte-specific fatty acid-binding

protein (aP2) mRNA expression and triglyceride levels in a dose-dependent manner at 10, 30, and 100 nM TBTCl, and linked to the $PPAR_{\gamma}$ pathway by induction of $PPAR_{\gamma}$ mRNA. Induction of adipocyte differentiation was also demonstrated by Grün et al. (2006) using histology and aP2 mRNA expression in mouse 3T3-L1 cells dosed with 10 and 100 nM TBT (presumably TBTCl, although compound, purity and source were not specified). The aP2 promoter contains an RXR:PPAR response element, implicating this pathway in the observed changes. In vitro exposure of mouse multipotent stromal stem cells to 5 and 50 nM TBT (presumably TBTCl although exact compound and purity not specified) increased adipogenesis, cellular lipid content, and expression of adipogenic genes (Fapb4, $PPAR_{\gamma}$, LEP) and decreased mRNA levels of the adipogenesis inhibitor Pref-1 (Kirchner et al. 2010). The adipogenic effects of TBT in this study were blocked by the addition of $PPAR_{\gamma}$ antagonists, suggesting that activation of $PPAR_{\gamma}$ mediates the effect of TBT on adipogenesis. TBT also induced $PPAR_{\gamma 2}$ and FABP4 protein expression in bone marrow multipotent mesenchymal stromal cells at concentrations >50 nM TBTCl, resulting in lipid accumulation and terminal adipocyte differentiation (Yanik et al. 2011). Interestingly, Belcher et al. (2014) found that TBT-induced human $PPAR_{\gamma}$ in a Chinese Hamster Ovary (CHO) cell line at 1 nM TBTCl and higher concentrations, but exhibited an "inverted U" dose-response curve, with maximal induction at 100 nM TBTCl. The cause of the loss in functional reporter gene activity was unclear.

Further evidence for the critical role for RXR in mediating effects of TBT comes from in vitro studies of effects on thyroid hormone receptors. Using thyroid hormone-responsive HepG2 cells, Sharan et al. (2014) demonstrated that TBTCl treatment induced a dose-dependent decrease in tri-iodothyronine (T3)-induced thyroid receptor (TR) transactivation and altered the expression of TRβ and its co-regulators including SRC-1 and NCoR. Therefore, TBT acts as an antagonist to TRs and inhibits T3-mediated transcriptional activity. However, TRs can form heterodimers with other nuclear receptors, in particular with the RXR. RXR plays a role in both positive and negative gene regulation through thyroid response elements (LaFlamme et al. 2002). Given the potential for TBT to also activate RXR, and the importance of RXR in the negative transcriptional regulation of genes of the hypothalamo-pituitary axis by T3 (LaFlamme et al. 2002), this suggests that TBT's effects on the thyroid axis may involve multiple nuclear receptor pathways.

5.7.2 Physiological Responses

Grün et al. (2006) dosed 6-week-old male mice for 24 h with 0.3 mg/kg bw TBT (presumably TBTCl, although compound, purity and source were not specified) and examined expression of critical transcriptional mediators of adipogenesis such as RXR_{α}, $PPAR_{\gamma}$, C/EBP α/β/δ, and sterol regulatory element binding factor 1 (Srebf1) as well as known target genes of RXR_{α}:$PPAR_{\gamma}$ signaling from liver, epididymal adipose tissue and testis. TBT either had no effect, or weakly repressed RXR_{α} and $PPAR_{\gamma}$ transcription in liver and decreased RXR_{α}, $PPAR_{\gamma}$, C/EBPα and

-δ in adipose tissue and testis. C/EBPβ was strongly induced in liver and testis, but more weakly induced in adipose tissue. Proadipogenic transcription factor Srebf1 was also induced in adipose tissue. Fatty acid transport protein (Fatp) mRNA levels were up-regulated two- to three-fold in liver and epididymal adipose tissue but not testis by TBT. Additional up-regulation of genes associated with fatty acid synthesis was also noted and together, these gene expression data confirmed TBT as a potential adipogenic agent in vivo.

Thyroid-related effects of TBT have been reported in vivo in mammals (e.g., Decherf et al. 2010; Sharan et al. 2014). Thyrotropin-releasing hormone (TRH) production is controlled at the transcriptional level by T3 through TRs but also via RXR and $PPAR_{\Upsilon}$ (LaFlamme et al. 2002). Decherf et al. (2010) exposed Swiss wild-type mice to TBT through lactation after dams were gavaged with a single 40 mg TBTCl/kg dose, and examined effects in pups on hypothalamic expression of genes implicated in metabolism and regulated by T3. They found that TBT dose-dependently increased T3-independent transcription from the TRH promoter (i.e., transcription that is controlled through RXR), but had no effect on T3-dependent repression. However, the effect on T3-independent expression was not observed in pups whose mothers were exposed chronically to 0.5 mg/kg by gavage for 14 days. Additionally, this paper demonstrated that exposure to TBT has a two-pronged effect on transcription from the aromatase and tyrosine hydroxylase promoters—it significantly reduced T3-independent transcription but also abolished T3-dependent regulation, confirming the role of TBT as a T3 antagonist that had been reported in vitro. Although the exposure levels in this study were relatively high, the results lend important insights into the hypothalamic effects of TBT.

Thyroid system effects in mice have been demonstrated at significantly lower exposure levels. Swiss albino male mice exposed to three doses of TBTCl (0.5, 5 and 50 μg/kg/day) for 45 days showed hypothyroidal effects (Sharan et al. 2014). TBT exposure markedly decreased serum thyroid hormone levels, which correlated with down-regulation of thyroid peroxidase (TPO) and thyroglobulin (Tg) genes in the thyroid gland and augmented circulating thyroid stimulating hormone (TSH) levels and TSH receptor (TSHr) gene in the thyroid gland. In addition, Pax8, a thyroid-specific transcription factor (mRNA and protein) and sodium-iodide symporter (Slc5a5) (mRNA) were also down-regulated. Sharan et al. (2014) concluded that TBT induces hypothyroidism by suppressing transcriptional activity of thyroid-responsive genes and inhibiting T3 binding to thyroid receptors, thereby preventing recruitment of co-activators and corepressors on the promoters of target genes.

Zuo et al. (2014) exposed male KM mice for 45 or 60 days to 0.5, 5, and 50 μg/kg TBTCl orally administered by gavage once every 3 days, and examined effects on the pancreas, glucose homeostasis, and circulating steroid and thyroid hormone levels. Animals treated with TBT for 60 days exhibited elevated fasting plasma glucose levels and decreased serum insulin and glucagon. TBT treatment for 45 days resulted in a dose-dependent increase in testosterone levels and a decrease in 17β-estradiol levels in the testes and serum compared to the control. Serum T4 levels did not show significant alteration in the TBT-exposed group, while T3 levels

showed a reduction in the TBT-exposed group and severe damage of the thyroid gland was observed histologically in mice exposed to 50 μg/kg TBT. No histological damage was observed in the pancreas after TBT exposure for 45 days. However, the number of apoptotic cells in the pancreas increased significantly with dose. TBT treatment for 45 days resulted in a dose-dependent decrease in pancreatic ERα expression but not ERβ levels, and resulted in an elevation of AR expression. This study is the first to examine direct endocrine effects of TBT on the pancreas.

Kirchner et al. (2010) investigated effects of in utero exposure of mice to TBT on adipose-derived stromal stem cells (ADSCs). Pregnant dams received a single 0.1 mg/kg body weight dose of TBT (presumably TBTCl although exact compound and purity not specified) by gavage and stromal cells were isolated from white adipose tissue (WAT) of their 8-week-old pups. Cells from TBT-exposed mice showed increased adipogenic capacity and lipid accumulation, reduced osteogenic capacity, increased Fapb4 and $PPAR_{\Upsilon}$ mRNA expression, decreased adipogenesis inhibitor Pref-1 mRNA, hypomethylation of the promoter/enhancer region of the Fapb4 locus, and an increased number of preadipocytes in the cells. This study provided the first evidence that in utero exposure to TBT counteracts osteogenesis and induces preferential differentiation of ADSCs into adipocytes.

5.7.3 Organismal Effects

In mammals, in vivo exposure to TBT has been shown to cause reproductive and other apical effects, although early studies used relatively high exposure levels, at which the effects of TBT are unlikely to be through the RXR/PPAR pathway. Harazono et al. (1996) reported a higher rate of pregnancy failure in Wistar rats exposed to 12.2 and 16.3 mg TBTCl/kg body weight. In a follow-up study, female Wistar rats exposed to 8.1, 16.3, or 32.5 mg TBTCl/kg body weight (25, 50, or 100 μM/kg) on days 0 through 3 of pregnancy, or 8.1, 16.3, 32.5, or 65.1 mg/kg (25, 50, 100, or 200 μM/kg) on days 4 through 7 of pregnancy by gastric intubation, and their fetuses, exhibited significantly lower body weights at 16.3 and 32.5 mg/kg than controls (Harazono et al. 1998). Exposure to 16.3 mg/kg and higher produced a significant increase in the rate of implantation failure, and dosing at the same levels on days 4–7 of pregnancy caused a significant increase in the incidence of post-implantation loss (Harazono et al. 1998). The authors concluded that susceptibility to, and manifestation of, the antifertility effects of TBTCl vary with the gestational stage at the time of administration. Dosing of rat dams with TBT beginning on gestational day 8 by oral gavage caused a significant reduction of dam's body weight at 10 mg/kg body weight during gestation and postnatally (Cooke et al. 2008). At postnatal days 6 and 12, neonatal pup weights were reduced at this concentration. However, Cooke et al. (2008) also noted that at the lowest dose of 0.25 mg TBTCl/kg body weight, dam's body weight increased relative to controls. Similarly, Zuo et al. (2009) showed that exposure of male mice to TBTCl at 5 μg/kg body weight for 45 days resulted in an increase in body weight and hepatic steatosis accompanied by hyperinsulinemia, hyperleptinemia, and changes in several

metabolism-related hormones. The variation in body weight responses appears to reflect both life stages during exposure and dose, with exposure in utero likely predisposing the animal to increased adipose mass as it ages and high dose exposures resulting in body weight loses.

One of the first studies to examine the effects of in utero exposure to TBT on lipid homeostasis and adipogenesis was conducted by Grün et al. (2006) using pups from pregnant C57BL/6 mice, which were injected intraperitoneally daily from gestational day 12–18 with 0.05 or 0.5 mg/kg body weight TBT (presumably TBTCl, although compound, purity, and source were not specified). Histological examination demonstrated that TBT exposure caused a disorganization of hepatic and gonadal architecture in the pups at birth, and liver sections exhibited signs of steatosis. Adipose mass in 10-week-old TBT-treated males was significantly higher than in controls although no overt increases in body mass were noted.

Effects of in utero exposure to TBT on fetal gonad morphology have been reported in Sprague–Dawley rats (Kishta et al. 2007). Light microscopic evaluation found that the number of Sertoli cells and gonocytes was reduced in fetuses whose mothers were gavaged daily from days 0 to 19 or 8 to 19 of gestation with 20 mg TBTCl/kg. Likewise, large intracellular spaces between Sertoli cells and gonocytes and increased abundance of lipid droplets in the Sertoli cells were observed. Electron microscopy studies revealed abnormally dilated endoplasmic reticulum in Sertoli cells and gonocytes. In the ovaries, TBT (20 mg/kg, days 0–19; 10 mg/kg, days 8–19) reduced the number of germ cells by 44% and 46%, respectively. Kishta et al. (2007) also examined gonadal gene expression in the fetuses and found significant up-regulation of testicular genes related to stress response but no up-regulation of these genes in the ovary. In ovaries, down-regulation was noted of genes involved with signal transduction.

TBT resulted in early puberty and impaired estrous cyclicity in female mice exposed perinatally (1, 10, or 100 μg TBTCl/kg body weight/day from day 6 of pregnancy), although no effects on circulating sex steroids (E2 or T) were observed (Si et al. 2012). Reductions in body weight were also reported by Si et al. (2012). Identical exposures of pregnant mice to TBT dramatically decreased sperm counts and motility in male offspring but had limited effects on intratesticular and serum hormone levels, suggesting that altered expression of receptors rather than hormone levels may be involved (Si et al. 2013).

5.7.4 Life-Cycle Studies

A two-generation reproductive toxicity study was conducted in rats using dietary exposure to TBT to evaluate its effect on sexual development and the reproductive system (Ogata et al. 2001; Omura et al. 2001). Pregnant female rats were exposed throughout pregnancy until weaning to 5, 25, or 125 μg TBTCl/g diet [assuming adult female rats weigh ~150 g and eat 16 g of food per day (US EPA 1988), this equates to approximately 12, 60, and 300 μg/kg body weight/day]. F0 and F1 progeny were provided with the same TBTCl diet as their mothers. For males

(Omura et al. 2001), significant effects on monitored endpoints (reduced body weight, delayed eye opening, reduced testis, epididymis and ventral prostate weights, and decreased spermatid count) were observed primarily at 125 μg/g. Only minimal histological changes were observed in the testes. A dose-dependent increase in serum testosterone occurred only in the F1 rats, and serum E2 was affected only in the 125 μg/g groups of F1 and F2. As is commonly seen in high dose exposures, the data suggest that these results were primarily related to direct toxic effects of TBT rather than functioning through specific endocrine pathways. A similar conclusion was drawn from the results of the companion study on the female offspring of these rats (Ogata et al. 2001). Reproductive outcomes of dams (number and body weight of pups and percentage of live pups) and the growth of female pups (day of eye opening and body weight gain) were significantly decreased in the group exposed to 125 μg TBTCl/g diet.

Chamorro-García et al. (2013) conducted a follow-up study to the one by Kirchner et al. (2010), in which they exposed female C57BL/6J mice prior to conception and during pregnancy to 5.42, 54.2, or 542 nM TBT (presumably TBTCl although exact compound, source and purity not specified) in drinking water (equivalent to 0.53, 5.3, and 53 μg/kg/day) to determine whether prenatal exposure would affect subsequent generations (F1 = exposed in utero, F2 = exposed as germ cells, F3 = no exposure). Prenatal TBT exposure elicited striking transgenerational effects in males including increased white adipose tissue (WAT) depot weights, adipocyte size, and adipocyte number at most doses in the three generations. More modest changes were observed in females, yet most doses of TBT led to significant increases in WAT depot weight and adipocyte size in F1 and F2 animals. Effects on body weight were modest and not directionally consistent in both sexes. Quantitative PCR analysis of adipogenic markers in bone marrow-derived multipotent mesenchymal stem cells (MSC) revealed sharply increased expression of Zfp423 and Fabp4 and decreased expression of Pref-1, an inhibitor of adipocyte differentiation, in TBT males from all three generations. Results from female mice were similar, but with less pronounced changes in F2 and greater variability in F3 mice than in males. Osteogenic markers, ALP and Runx2, sharply decreased in F1 and F3 males and females but were primarily unchanged in F2. All three generations exhibited hepatic lipid accumulation and up-regulation of hepatic genes involved in lipid storage/transport, lipogenesis, and lipolysis. The Chamorro-García et al. (2013) results show that early-life exposure to TBT can have transgenerational effects on adipogenesis, at least through the F3 generation.

6 Adverse Outcome Pathway

The adverse outcome pathway (AOP) is a framework that summarizes existing information for a given biological pathway from the MIE, through various levels of biological organization (genetic, molecular, physiological, and organismal), and culminates with population-relevant results that can be used for ecological risk assessment. This framework was described by Ankley et al. (2010) and has been

utilized many times to highlight progressive linkages between receptor activation and population-relevant outcomes.

The present review provides the necessary information needed to populate the various AOP components, which are displayed in Fig. 3. It should be noted that this is a putative and most likely incomplete AOP that was drafted based on existing information and our current ability to interpret the available data. In order to obtain the complete AOP, far more work is needed to fill in the gaps between MIEs and key events (KE) across the different taxa and life stages (applicability domains); even more work is required to obtain a quantitative AOP as it is likely that different pathways are operating and their LOECs may be different between applicability domains. Since TBT can trigger more than one AOP, the potential for an operational AOP network is substantial and elucidation on whether this network is converging, diverging or independent in terms of adversity will be needed. All the information captured in Fig. 3 originates from invertebrate (primarily molluscan) studies, although certain pathways have been confirmed in other taxa. Besides the putative and incomplete nature of the AOP presented here, it is almost certain that the critical pathway appears to be initiated by the RXR and RXR/PPAR interactions, to include imposex as a key event and to result in complete reproductive failure and decline population trajectory.

The full AOP can only be completed when some basic aspects of endocrinology of invertebrate species, that are currently largely unknown, become available. The same holds true for the RXR and RXR/PPAR modulated pathways in vertebrate species, although some information has recently become available (Tonk et al. 2015). Nevertheless, it should be noted that unlike the majority of the currently available AOPs in the context of chemical perturbations, the adverse outcome of TBT exposure at population level is well established at least for molluscan species as the numerous publications on population extinctions globally testify.

7 Species Sensitivity Distribution (SSD) for Toxic Effects in Aquatic Organisms

7.1 Water Exposure SSDs

Species sensitivity distributions (SSDs) have been derived using apical, population-relevant data (reproductive outcomes, including fertilization, embryonic development, hatching, larval and juvenile growth, as well as sex ratio) mainly from long-term aqueous exposure studies matching Levels 4 and 5 in the OECD CFEDTA (see Tables 2 and 3 of the Annexes). These SSDs do not necessarily include all relevant species and endpoints and are not expected to be comprehensive. However, they have been built using robust endpoints from reliable studies, and therefore accurately reflect the sensitivity of major groups of aquatic organisms. Two SSDs have been constructed—one using LOECs (Fig. 4) and the other NOECs (Fig. 5) from chronic studies—using the ETX 2.1 software of the Netherlands National Institute for Public Health and the Environment (RIVM) (van Vlaardingen et al. 2014).

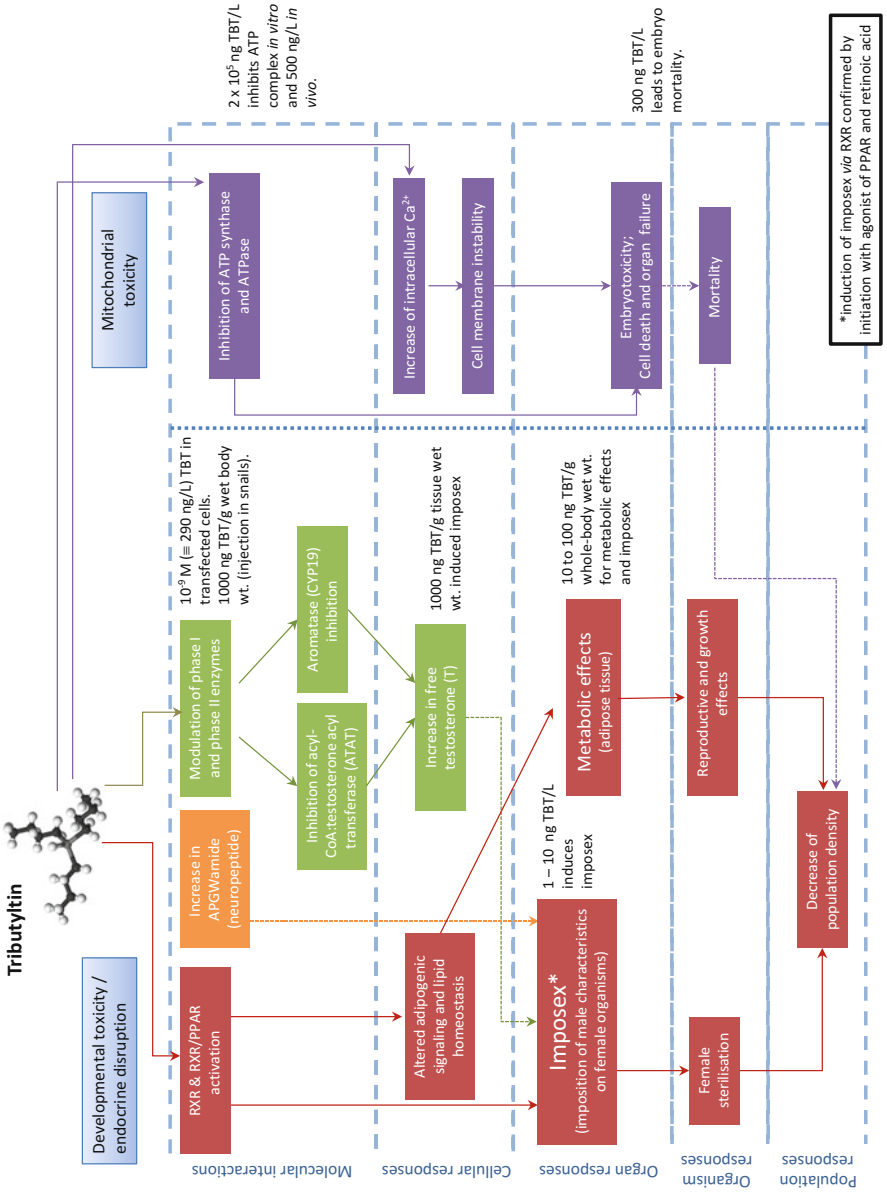
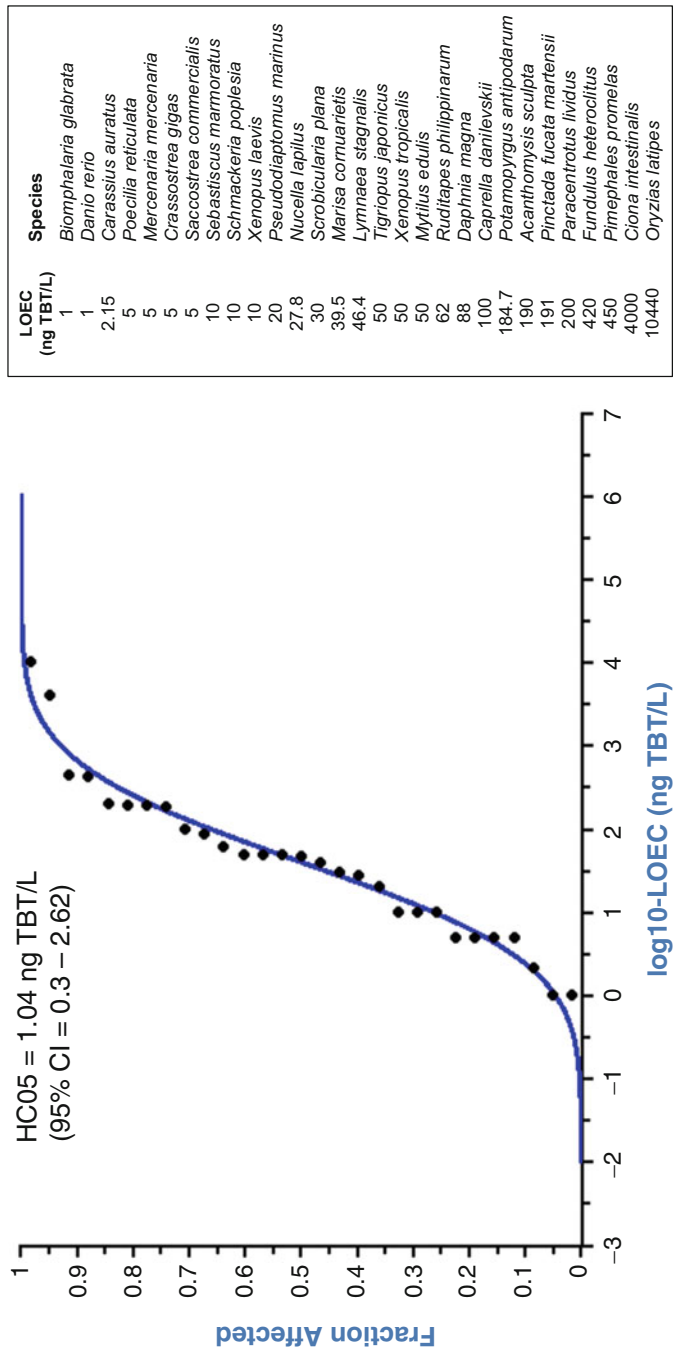
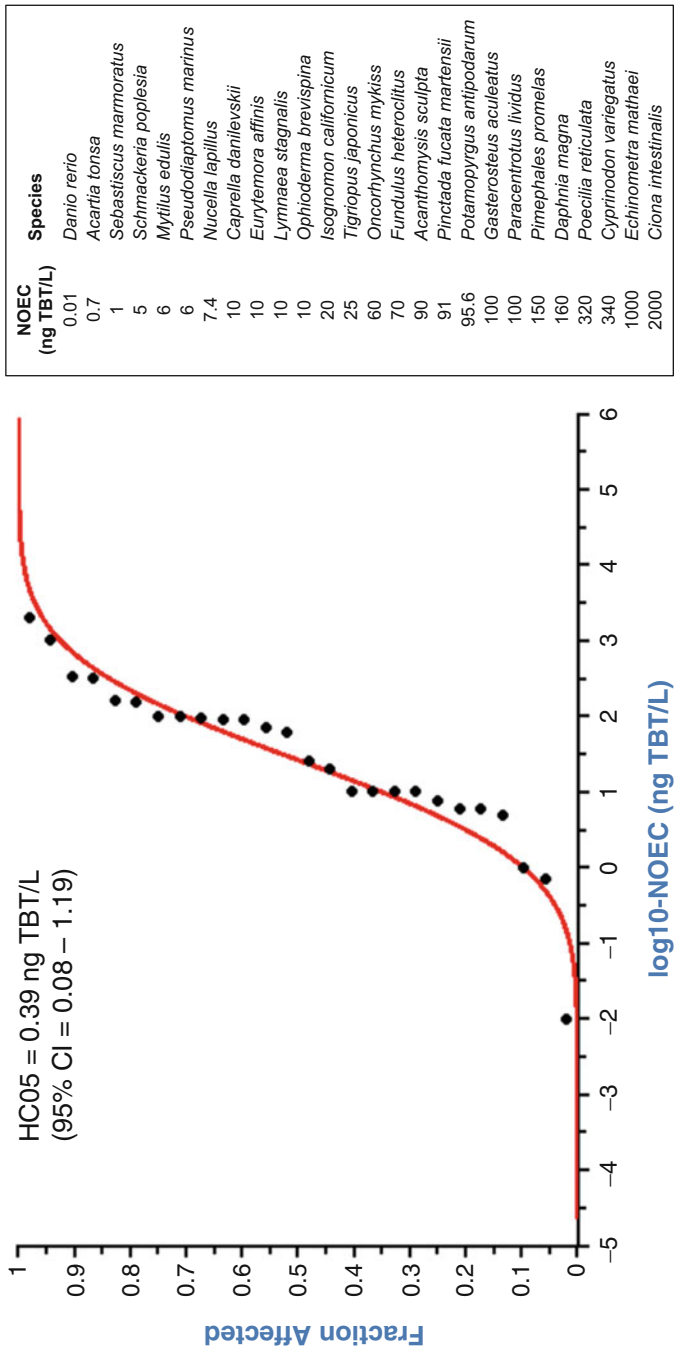


Fig. 3 Proposed adverse outcome pathway (AOP) for TBT toxicity



LOEC (ng TBT/L)	Species
1	*Biomphalaria glabrata*
1	*Danio rerio*
2.15	*Carassius auratus*
5	*Poecilia reticulata*
5	*Mercenaria mercenaria*
5	*Crassostrea gigas*
5	*Saccostrea commercialis*
10	*Sebastiscus marmoratus*
10	*Schmackeria poplesia*
10	*Xenopus laevis*
20	*Pseudodiaptomus marinus*
27.8	*Nucella lapilus*
30	*Scrobicularia plana*
39.5	*Marisa cornuarietis*
46.4	*Lymnaea stagnalis*
50	*Tigriopus japonicus*
50	*Xenopus tropicalis*
50	*Mytilus edulis*
62	*Ruditapes philippinarum*
88	*Daphnia magna*
100	*Caprella danilevskii*
184.7	*Potamopyrgus antipodarum*
190	*Acanthomysis sculpta*
191	*Pinctada fucata martensii*
200	*Paracentrotus lividus*
420	*Fundulus heteroclitus*
450	*Pimephales promelas*
4000	*Ciona intestinalis*
10440	*Oryzias latipes*

Fig. 4 Species sensitivity distribution (SSD) constructed with sublethal population-relevant LOEC values for TBT in 29 aquatic species. See Table 2 of the Annexes for the endpoint value and description for each species



NOEC (ng TBT/L)	Species
0.01	*Danio rerio*
0.7	*Acartia tonsa*
1	*Sebastiscus marmoratus*
5	*Schmackeria poplesia*
6	*Mytilus edulis*
6	*Pseudodiaptomus marinus*
7.4	*Nucella lapillus*
10	*Caprella danilevskii*
10	*Eurytemora affinis*
10	*Lymnaea stagnalis*
10	*Ophioderma brevispina*
20	*Isognomon californicum*
25	*Tigriopus japonicus*
60	*Oncorhynchus mykiss*
70	*Fundulus heteroclitus*
90	*Acanthomysis sculpta*
91	*Pinctada fucata martensii*
95.6	*Potamopyrgus antipodarum*
100	*Gasterosteus aculeatus*
100	*Paracentrotus lividus*
150	*Pimephales promelas*
160	*Daphnia magna*
320	*Poecilia reticulata*
340	*Cyprinodon variegatus*
1000	*Echinometra mathaei*
2000	*Ciona intestinalis*

Fig. 5 Species sensitivity distribution (SSD) constructed with population-relevant NOEC values for TBT in 26 aquatic species. See Table 3 of the Annexes for the endpoint value and description for each species

When several biological endpoints were available for one given species, only the lowest LOEC and/or NOEC was used, to ensure that each species appeared only once in the SSD. In some cases, the LOECs are unbounded, so the true effect concentration could be much lower. The NOEC is also sometimes significantly lower than the LOEC, depending on test design (i.e., some may be over-precautionary). By assessing the distribution of both LOECs and NOECs, these factors have been ignored for the purposes of this analysis. For both SSDs, the goodness-of-fit was acceptable (significance level: 0.01–0.1), as assessed using the Anderson-Darling, Kolmogorov-Smirnov, and Cramer von Mises tests for normality.

As a result from the NOEC-based SSD, 0.39 ng/L (95%-CI: 0.08–1.19 ng/L) was determined to be the HC5 (the predicted concentration that affects 5% of the species in the distribution), whereas the HC5 based on LOEC values was 1.04 ng/L (95%-CI: 0.3–2.62 ng/L). The NOEC-based HC5 value is close to current EU regulatory thresholds (e.g., 0.2 ng/L; EU 2005), which incorporates a safety factor and so should be treated with caution. For comparison, the USEPA ambient water quality criterion (seawater chronic) has been set to 7 ng/L (US EPA 2003).

An interesting feature of this sensitivity analysis is that some fish and other invertebrate species are more sensitive to TBT than caenogastropod molluscs, in particular some copepods (*Acartia tonsa*, *Schmackeria poplesia Pseudodiaptomus marinus*) and the fish *Danio rerio* and *Sebastiscus marmoratus*. The lowest endocrine-sensitive LOEC identified in this review was 0.1 ng TBT/L for a male-biased sex ratio change and abnormal sperm in zebrafish (McAllister and Kime 2003). Our analysis found that study convincing, well designed, and described. Aqueous concentrations of TBT in aquaria for the highest dose (100 ± 5 ng TBT/L) were verified by gas flame atomic absorption spectrophotometry while other doses were below the detection limits. Because this is the lowest value, it sets the lower limit concentration for the TBT risk assessment for fish and also for molluscs and other invertebrate species.

Such a high sensitivity of zebrafish sex ratio to TBT is consistent with the fact that TBT inhibits aromatase, thus increasing the level of free testosterone to which zebrafish appear to be extremely sensitive (Holbech et al. 2006; Örn et al. 2006; OECD 2012b). There is therefore a biological plausibility that links the molecular initiating event (aromatase inhibition) to the key event (increased levels of free testosterone) subsequently leading to an apical effect (male-biased sex ratio), which is meaningful for inferring potential impact at the population level. Such causality relationships were thoroughly described by Matthiessen and Weltje (2015) for azoles compounds, which also act as aromatase inhibitors.

Comparison of the potencies of known aromatase inhibitors, such as prochloraz, with TBT requires an in-depth analysis of the available data. However, this is somehow out of the scope of this review. Nevertheless, it appears that, with an IC_{50} around 0.2 μM (Cooke 2002), TBT is a weaker aromatase inhibitor in vitro as compared to prochloraz (IC_{50} = 0.04 μM; Vinggaard et al. 2000). In contrast, in vivo (zebrafish fish sexual development test), a dramatic shift in potencies was observed with TBT affecting sex ratio towards males at 1 ng/L (McAllister and

Kime 2003) whilst prochloraz shifted sex ratio towards males at 202 µg/L (Kinnberg et al. 2007). It is highly unlikely such a large difference stems from slightly different exposure conditions. Hence, it could be speculated that TBT exerts its actions on vertebrate sexual development via additional mechanisms that enhance the effects of aromatase inhibition alone. On the other hand, direct comparison is hindered by the fact that prochloraz along with other pesticides have additional endocrine modulating properties (i.e., androgen and estrogen receptor interactions). A thorough review of these data is required before firm conclusions can be made on the primary adverse outcome pathway of TBT in vertebrates.

There were various effects (including in vitro changes in genomic markers and enzyme activities) in other fish species within at least one order of magnitude of the results obtained by McAllister and Kime (2003) but these endpoints and LOEC values for zebrafish stand out as the most ecotoxicologically relevant. This suggests that the endpoints used for other fish studies (often growth) were not the most relevant. However, the dramatic effect observed in zebrafish may be restricted to fish species that share the same pattern for sexual differentiation rather than all fish. Zebrafish gonads initially develop as ovaries, however in male fish, the ovarian tissue degenerates and the testis develop (Maack and Segner 2003). This period of juvenile hermaphroditism (Takahashi 1977) may explain the increased sensitivity of the sex ratio endpoint after exposure to AR agonists and aromatase inhibitors during critical developmental windows.

7.2 Tissue Residue-Based Analysis

A given ambient toxicity metric (e.g., EC_{50} or LOEC) that is based on aqueous or sediment concentrations can result in a range spanning orders of magnitude for different species. The equivalent tissue residue toxicity metric (e.g., ER_{50} or LOER) often exhibits lower variability (Meador 1997). This has been observed for a large variety of taxa and chemicals, which has been discussed in many publications (Meador 2006; Meador et al. 2008; McElroy et al. 2011) and is known as the tissue-residue approach for toxicity assessment. TBT provided one of the first examples of the utility for this approach based on a large database of tissue residue toxicity data for mortality and reduced growth. The analysis of TBT toxicity has expanded from evaluating these high dose endpoints to endocrine-related responses in invertebrates and fish responding to very low environmental concentrations. While some of the datasets are limited, they do indicate a relatively consistent response among species for a given endpoint and whole-body tissue concentration (Meador 2011). Widely variable toxicokinetics among species is the main factor responsible for the high interspecies variability for a given toxicity metric based on external dose. Consequently, when internal dose is used to determine a toxicity value, toxicokinetic differences are not a confounding factor, which results in greatly reduced variability among species response values (Meador et al. 2008; McCarty et al. 2011).

An analysis of the imposex endpoint as a function of whole-body tissue concentrations is shown in Fig. 6. The program SSDMaster (Rodney and Moore 2008) was

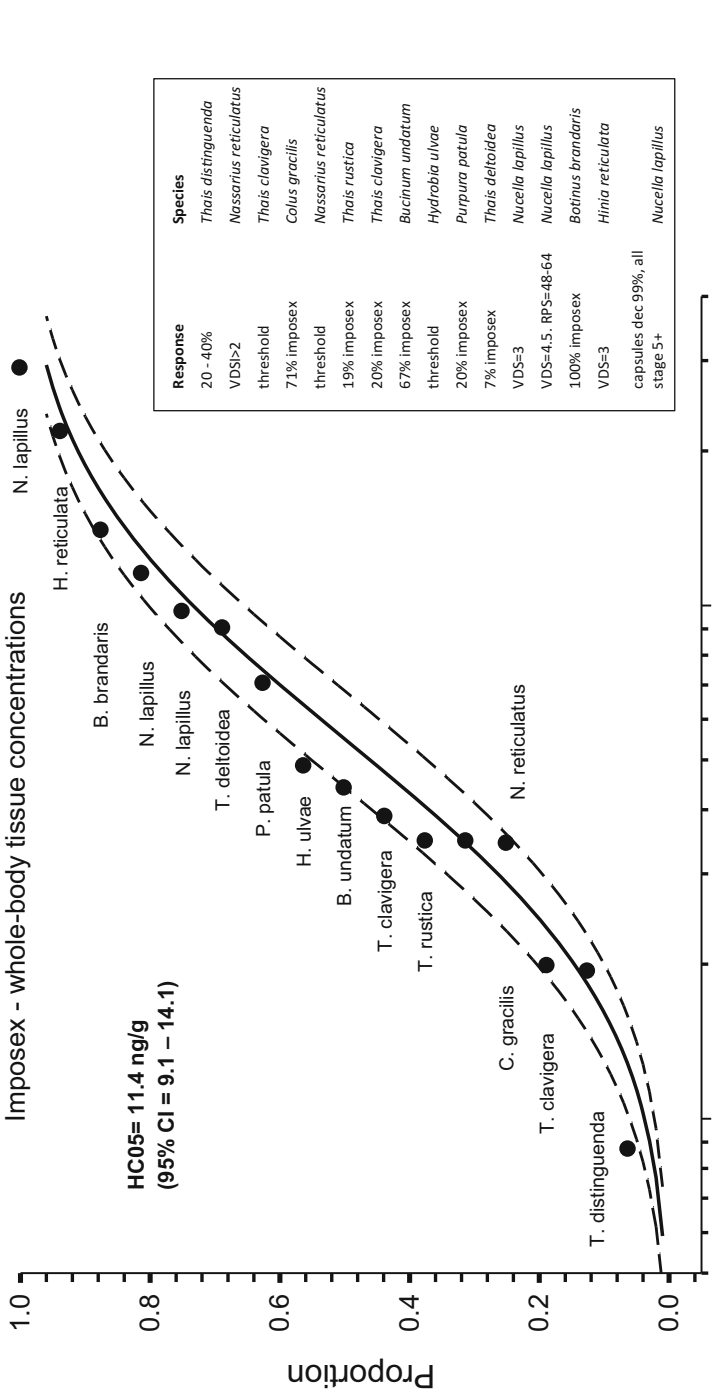


Fig. 6 Cumulative distribution function (CDF) constructed with tissue TBT concentrations (as ng TBT/g wet weight) that induce imposex in Caenogastropod molluscs

used to generate the data for this plot. The cumulative distribution function (CDF) shows that the sensitivity of one given species may vary according to the imposex stage and TBT tissue burden. These data indicate a relatively narrow range of concentrations for the imposex response spanning from threshold to 100% induced. Based on these data, the hazard concentration resulting in low level effects was determined to be 11.4 ng TBT/g wet weight (95% CI = 9.1–14.1). This concentration characterizes a low level response; hence, a safety factor may be needed to determine the potential "no effect" level. The tissue concentrations in this CDF have utility in assessing population fitness for these gastropods in the field.

8 Sources of Uncertainty, Data Gaps, and Confounding Issues

8.1 Transgenerational Effects

TBT is highly bioaccumulative and maternal transfer to eggs has been demonstrated (e.g., Inoue et al. 2006; Ohji et al. 2006). Effects can occur over multiple generations in some invertebrate species, e.g. chironomids (Lilley et al. 2012) and copepods (Huang et al. 2006), as well as in fish, birds, and mammals. However, it is not clear whether or not this is exclusively linked to endocrine-mediated mechanisms.

8.2 Sensitive Species

The general perception is that molluscs are the most sensitive taxonomic group to TBT exposure due to the observation of imposex in wild species at low environmental concentrations. However, the present analysis determined that a number of fish species and other invertebrate species (e.g., copepods) have similar or greater levels of sensitivity when considered in terms of population-relevant responses.

8.3 Potency

With respect to endocrine-mediated effects, TBT is highly potent as it can act in aquatic organisms at levels of parts per trillion (ng/L) and lower (Figs. 4 and 5), and low ng/g tissue concentrations (Fig. 6), whereas lethal toxicity occurs at much higher concentrations. Thus, the HC5 value derived from an SSD based on LOECs for mortality (Fig. 7) is ten times higher than the HC5 estimated from LOEC for sublethal effects (Fig. 4). In the copepod *Acartia tonsa*, the NOEC values for

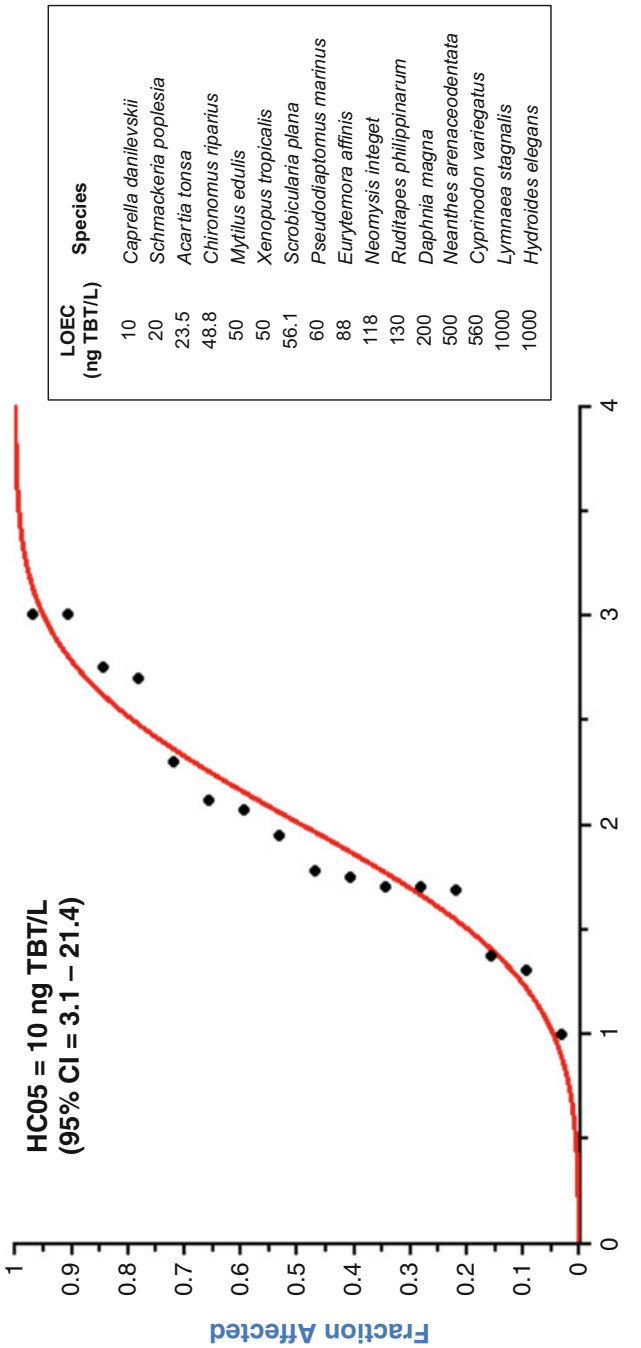


Fig. 7 Species sensitivity distribution (SSD) constructed with TBT LOEC values for mortality in 16 most sensitive aquatic species. Data that were used to construct this SSD are given in Table 4 of the Annexes). The goodness-of-fit was acceptable (significance level: 0.01–0.1), as assessed using the Anderson-Darling, Kolmogorov-Smirnov, and Cramer von Mises tests for normality

reproductive effects and mortality are 0.7 and 11 ng/L, respectively (Kusk and Petersen 1997).

Potency of TBT is also shown at the molecular level where molecular initiating events are elicited at concentrations that are several hundred times lower than those causing basal toxicity (Fig. 3).

8.4 Non-monotonic Dose-Response or Lack of a Threshold Dose

Inverted U-shape responses to TBT have been observed for some endpoints, depending on the concentration, mainly involving gene expression studies (e.g., Mortensen and Arukwe 2007; Kortner et al. 2010; Morales et al. 2013; Pascoal et al. 2013). From in vivo studies, there is also some evidence that TBT has been shown to display an inverted U-shape response for several endpoints. A good example is the impact of TBT on body weight in fish and mammals (Cooke et al. 2008; Meador et al. 2011; Si et al. 2011). Meador et al. (2011), for example, found that TBT exposure in fish enhanced growth and lipogenesis at low doses and inhibited growth and reduced lipid content at high doses; this was attributed to two modes of action operating at different doses. Most likely, the cause of a non-linear response for TBT is different dose-dependent mechanisms of action. TBT is known to be an uncoupler of oxidative phosphorylation and directly affects ATPase (ATP-synthase). This enzyme that plays an important role for providing cellular energy is located within the mitochondrial membrane and consists of two regions: the Fo section, embedded within the membrane and the F_1 section, outside the membrane but inside the matrix of the mitochondria. TBT interacts with the Fo section of ATPase. When exposures occur at higher doses, this is the most likely MeOA that causes reduced growth and death. Baseline toxicity (narcosis) is not possible because all species die at about 10^4 ng/g whole-body concentration, far below baseline doses. Hence, the low dose effects are definitely due to RXR-PPAR binding but high dose responses are due to mitochondrial dysfunction. Of course, the U-shaped response will depend on the endpoint; it is highly plausible for metabolic effects. The question is whether this non-linear behavior will occur for other responses. Such non-linear concentration-response should be considered with caution regarding the magnitude and consistency of the changes. It is important to determine whether or not these responses are significant, and if they consistently occur in different species. One should also consider whether U-shaped dose-responses are a result of endocrine-mediated perturbations, multiple mechanisms of action, or adaptive process (see Parrott et al. 2017, and the references therein). They also may relate to exposure artifacts (e.g., exceeding chemical solubility, erroneous doses, or failure of chemical delivery systems).

9 Areas for Future Research

Although TBT has now been recognized as an EDC at a global scale, if its ED properties were originally assessed at low concentrations using a limited group of USEPA Tier-1 tests that did not include the AMA, further testing may not have been triggered. The current Tier-1 tests cover primarily three hormonal pathways (EAT) and as such do not target the primary endocrine mode of action of TBT, via RXR/PPAR activation. Based on the literature reviewed here, TBT would elicit positive results in the steroidogenesis and aromatase assays only at levels much higher than those needed to elicit an endocrine effect via the RXR/PPAR interactions. The lack of testing for other hormonally and metabolically important nuclear receptors such as PPAR and RXR under both the US and OECD testing batteries has been recognized as a deficiency and prioritized for assay development by OECD (OECD 2012a). The results of the present review underscore this need. However, we recognize that assays for RXR/PPAR activity may be more difficult to develop and validate than those for other nuclear receptors such as ER and AR, due to the complex nature of their activation, the formation of homo- and heterodimers with other nuclear receptors, and the permissive/non-permissive nature of downstream effects. In other words, a different platform than the current transactivation assays (e.g., one that allows assessing cross-talk between these receptors) may be needed for a full understanding of perturbations stemming from chemical interactions with these receptors.

In addition to new low-tier testing that goes beyond EAT, suitable higher tier tests should also be developed in order to assess the plethora of biological effects that involve targets other than EAT. To this end, the new OECD Test Guidelines (TG242 and TG243) on molluscan reproductive toxicity are an important development, although such tests do not discriminate between ED and non-ED mediated mechanisms. However, standardized partial or full life-cycle tests should also be developed for additional invertebrate phyla such as annelids and echinoderms as they are not only numerous, but also ecologically important taxonomic groups with a largely unknown endocrine system.

When designing a testing strategy for chemicals that are suspected to act via the endocrine system, ecotoxicologists should incorporate the lessons learned from TBT, as they are both numerous and important. The first relates to apparent species sensitivity. Previously, molluscs were assumed to be the most sensitive group to TBT, primarily due to overt population effects. However, our analyses demonstrated that certain fish species are equally if not more sensitive to its effects. Likewise, the body burden of a chemical should be taken into account especially when the apparent sensitive species are filter feeders. In many cases, these species exhibit elevated bioaccumulation because of higher rates of uptake and lower rates of metabolism compared to other species. Tissue residue toxicity metrics also vastly improve the characterization of toxic responses because the inherent variability in toxicokinetics found among species is incorporated and accounted for.

Another important aspect relates to species extrapolation, particularly when fundamental aspects of endocrine control are largely unknown. TBT is not the only chemical with observed effects in invertebrate species that were subject to erroneous interpretations based on vertebrate endocrinology. The same assumption was made for the role of estrogens on bivalve vitellogenesis (Gagné et al. 2001), which resulted in numerous research programs globally attempting to use bivalve molluscs as model species for studying effects of vertebrate steroids (Scott 2012, 2013). The presence of an estrogen-like receptor in their genome cemented this assumption without any functional characterization of this receptor (Kishida et al. 2005). Only recently this assumption was proven incorrect (Morthorst et al. 2014), highlighting yet again the importance of fundamental knowledge before a sound testing strategy is in place.

Finally, another important lesson learned from TBT is that care should be taken when dealing with chemicals that display multiple mechanisms of action; the actual number of these is unknown but they do exist and can lead to incorrect interpretations of experimental and field data. Retrospective analysis of the TBT data clearly indicates a dual mode of toxicity (low versus high doses) that is a function of different MeOA. This highlights the need for comprehensive testing at different levels and using different species prior to interpretation of data.

10 Conclusions and Recommendations

Environmental TBT concentrations measured post-2008 show that exposure may still occur in the range 0.1–8 ng/L (mean = 0.3 ng/L) in representative European surface waters (UK Environment Agency, pers. com. 2016) and in other locations (e.g., Kim et al. 2014; Ho et al. 2016), suggesting that there is still an environmental risk from legacy contamination. Risks may be higher in regions of the world with less effective enforcement. TBT was introduced on the market in the early 1960s, at a time when regulatory assessment of chemicals was at its infancy. Using deliberate "retrospective thinking," and considering the information gathered in this case study, one important question arises: would TBT be identified as an ED using current screening and testing methods?

Typical endocrine responses are elicited by compounds that mimic estrogen, androgen, and thyroid pathway hormones and act via various nuclear receptors. Tributyltin is not considered a classic endocrine disruptor, because it impacts reproductive and metabolic pathways primarily through interaction with the retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPARγ) nuclear receptors.

Using assays recommended in the OECD CFEDTA, TBT was shown to alter the sex-ratio and to induce sperm abnormality in the Fish Sexual Development Test (FSDT) with zebrafish (McAllister and Kime 2003), and to delay frog development in the AMA (Shi et al. 2014), at low concentrations (0.1 and 10 ng/L, respectively). It also appeared positive in a FETAX-like assay (Guo et al. 2010). Based upon these

findings, TBT would be identified as acting on endocrine pathways, although the specific MeOA (RXR and/or PPAR) would remain unknown. However, refinements to ToxCast™ now allow the identification of endocrine molecular initiating events through RXR and/or PPAR pathways. TBT activity for in vitro RXR and PPAR assays typically occurs at levels far less than those for baseline toxicity.

Interestingly, a more thorough evaluation of the available data clearly shows that TBT is highly toxic to a variety of aquatic taxa. Through a comparative analysis of the potency of TBT in various aquatic species, our review highlights the observation that fish are as sensitive, or more so, compared to molluscs when based on water exposure. This is an important conclusion because molluscs were long recognized as uniquely sensitive to this compound. TBT's precise MeOA is still incompletely understood but may include link/cross-talk between PPARs (i.e., carbohydrate, lipid, protein metabolism), RXRs (i.e., development), thyroid (growth) and even sex determination and differentiation pathways; the latter pathways may be stronger affected by TBT exposure in species where environmental factors play a significant role in determining sex ratios (e.g., zebrafish).

Current screening and assessment methodologies are able to identify TBT as a potent endocrine disruptor with a high environmental risk. If those approaches were available when TBT was introduced to the market, it is likely that its use would have been regulated sooner, thus avoiding the detrimental effects on marine gastropod populations and communities as documented over several decades.

This retrospective evaluation of TBT, a very potent endocrine disruptor in vertebrates and invertebrates, should serve as an example demonstrating how shortfalls within the framework of chemical toxicity evaluation can result in under-protective regulatory assessment. Nowadays, the assays included in the OECD Conceptual Framework, including those recently developed on gastropod molluscs would likely recognize TBT as a chemical of concern with respect to endocrine disruption, although its mechanism of action and potency across taxonomic groups would remain largely unknown. Reflective analysis of well-studied, but potentially misunderstood contaminants, such as TBT, provides important lessons that should serve as a guiding principle for future studies and refinements of assessment protocols.

11 Summary

Tributyltin (TBT) has been recognized as an endocrine disrupting chemical (EDC) for several decades. However, only in the last decade, was its primary endocrine mechanism of action (MeOA) elucidated—interactions with the nuclear retinoid-X receptor (RXR), peroxisome proliferator-activated receptor γ (PPARγ), and their heterodimers. This molecular initiating event (MIE) alters a range of reproductive, developmental, and metabolic pathways at the organism level. One of the most important lessons learned from years of research on TBT concerns apparent species sensitivity. Several aspects such as the rates of uptake and elimination, chemical

potency, and metabolic capacity are all important for identifying the most sensitive species for a given chemical, including EDCs. As recognized for many years, TBT-induced responses are known to occur at very low concentrations for molluscs, a fact that has more recently also been observed in fish species. This review explores the MeOA and effects of TBT in different species (aquatic molluscs and other invertebrates, fish, amphibians, birds and mammals) according to the OECD Conceptual Framework for Endocrine Disruptor Testing and Assessment (CFEDTA). The information gathered on biological effects that are relevant for populations of aquatic animals was used to construct Species Sensitivity Distributions (SSDs) based on No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs). Fish appear at the lower end of these distributions, showing that they are as sensitive as molluscs, and for some species, even more sensitive. Concentrations in the range of 1 ng/L for water exposure (10 ng/g for whole-body burden) have been shown to elicit endocrine-type responses, whereas mortality occurs at water concentrations ten times higher. Current screening and assessment methodologies as compiled in the OECD CFEDTA are able to identify TBT as a potent endocrine disruptor with a high environmental risk for the original use pattern. If those approaches had been available when TBT was introduced to the market, it is likely that its use would have been regulated sooner, thus avoiding the detrimental effects on marine gastropod populations and communities as documented over several decades.

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Disclaimer

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Conflict of Interest

The authors declare that they have no conflict of interest.

Annexes

Table 1 Updated OECD conceptual framework for testing and assessment of endocrine disrupters (OECD 2012a, b)

Level	Mammalian and non-mammalian toxicology
1	• Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability
Existing data and non-test information	• All available (eco)toxicological data from standardized or non-standardized tests
	• Read across, chemical categories, QSARs and other in silico predictions, and ADME model predictions
2	• Estrogen or androgen receptor binding affinity

(continued)

Level	Mammalian and non-mammalian toxicology	
In vitro assays providing data about selected endocrine mechanism(s)/pathways(s)	• Estrogen receptor transactivation (TG 455–TG 457)	
	• Androgen or thyroid transactivation (if/when TGs are available)	
	• Steroidogenesis in vitro (TG 456)	
	• MCF-7 cell proliferation assays (ER ant/agonist)	
	• Other assays as appropriate	
	Mammalian toxicology	Non-mammalian toxicology
3 In vivo assays providing data about selected endocrine mechanism(s)/pathway(s)[a]	• Uterotrophic assay (TG 440)	• Xenopus embryo thyroid signaling assay (when/if TG is available)
		• Amphibian metamorphosis assay (TG 231)
	• Hershberger assay (TG 441)	• Fish reproductive screening assay (TG 229)
		• Fish screening assay (TG 230)
		• Androgenized female stickleback screen (GD 140)
4	• Repeated dose 28-day study (TG 407)	• Fish sexual development test (TG 234)
In vivo assays providing data on adverse effects on endocrine relevant endpoints[b]	• Repeated dose 90-day study (TG 408)	• Fish reproduction partial life-cycle test (when/if TG is available)
	• One-generation reproduction toxicity study (TG 415)	• Larval amphibian growth & development assay (when TG is available)
	• Male pubertal assay (see GD 150, C4.3)[c]	
	• Female pubertal assay (see GD 150, C4.4)[c]	• Avian reproduction assay (TG 206)
		• Mollusc reproduction test (TG 242–TG 243, adopted 2016)[d]
	• Intact adult male endocrine screening assay (see GD 150, Annex 2.5)	• Chironomid toxicity test (TG 218–219)[d]
		• Daphnia reproduction test (with male induction) (TG 211)[d]
	• Prenatal developmental toxicity study (TG 414)	• Earthworm reproduction test (TG 222)[d]
	• Chronic toxicity and carcinogenicity studies (TG 451-3)	• Enchytraeid reproduction test (TG 220)[d]
	• Reproductive screening test (TG 421 if enhanced)	• Sediment water Lumbriculus toxicity test using spiked sediment (TG 225)[d]
	• Combined 28-day/reproductive screening assay (TG 422 if enhanced)	• Predatory mite reproduction test in soil (TG 226)[d]
		• Collembolan reproduction test in soil (TG 232)[d]
	• Developmental neurotoxicity (TG 426)	

(continued)

Level	Mammalian and non-mammalian toxicology	
5	• Extended one-generation reproductive toxicity study (TG 443)[e]	• Medaka extended one-generation reproduction test (MEOGRT) (TG 240)
In vivo assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism[b]	• Two-generation reproduction toxicity study (TG 416 most recent update)	• FLCTT (fish life-cycle toxicity test) (when TG is available)
		• Avian two-generation reproductive toxicity assay (when TG is available)
		• Mysid life-cycle toxicity test (when TG is available)[d]
		• Copepod reproduction and development test (when TG is available)[d]
		• Sediment water chironomid life-cycle toxicity test (OECD TG 233)[d]
		• Mollusc full life-cycle assays (when TG is available)[d]

[a]Some assays may also provide some evidence of adverse effects
[b]Effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms
[c]Depending on the guideline/protocol used, the fact that a substance may interact with a hormone system in these assays does not necessarily mean that when the substance is used it will cause adverse effects in humans or ecological systems
[d]At present, the available invertebrate assays solely involve apical endpoints which are able to respond to some endocrine disrupters and some non-EDs. Those in Level 4 are partial life-cycle tests, while those in Level 5 are full- or multiple life-cycle tests
[e]The Extended one-generation reproductive toxicity study (TG 443) is preferable for detecting endocrine disruption because it provides an evaluation of a number of endocrine endpoints in F1 juvenile and adult, which are not included in the two-generation study (TG 416 adopted 2001)

Table 2 LOEC (lowest observed effect concentration) of TBT for population-relevant endpoints in aquatic organisms

Species	Test conditions	Biological endpoint	LOEC (ng TBT/L)	Reported as[a]	Reference
Amphibians					
Xenopus laevis	AMA (stage 51 tadpoles)/CAMA (stage 46 tadpoles)	Embryo body length and development	10.00	nom	Shi et al. (2014)
Xenopus tropicalis	FETAX-like test	Tadpole development	50.00	nom	Guo et al. (2010)
Fish					
Carassius auratus	Adults, 54 days (semi-static exposure)	Body weight, swimming activity	2.15	mm	Zhang et al. (2016)
Danio rerio	Full life-cycle test	Sperm abnormality 3–5 months post-exposure	0.10	nom	McAllister and Kime (2003)
Danio rerio	Larvae, 0–70 days post-hatch	Sex ratio	0.10	nom	McAllister and Kime (2003)
Fundulus heteroclitus	Full life-cycle test	Sex ratio F_1	420.00	mm	Mochida et al. (2010)
Fundulus heteroclitus	Full life-cycle test	Time to hatch F_1	750.00	mm	Mochida et al. (2010)
Oryzias latipes	Embryos	Hatching success	10,440.00	mm?	Bentivegna and Piatkowski (1998)
Pimephales promelas	Early life-stage test, 32 days	Fry growth and weight	450.00	mm?	Brooke et al. (2003)
Poecilia reticulata	Male adults, 28 days	Reproductive behavior	5.00	nom	Tian et al. (2015)
Sebastiscus marmoratus	Embryos (gastrula stage), 144 h	Hatchability	10.00	nom	Zhang et al. (2011)
Crustaceans					
Acanthomysis sculpta	Life-cycle test	Reproduction	190.00	mm	Davidson et al. (2003)
Caprella danilevskii	Full life-cycle test	Sex ratio	100.00	nom	Ohji et al. (2002)
Daphnia magna	F_0 third-instar juveniles (3 days) and F_1 egg provisioning stage	Reproduction	88.00	mm	Jordão et al. (2015)
Daphnia magna	Adults, 21 days	Offspring/female	2500.00	nom	Oberdorster et al. (1998)

(continued)

Table 2 (continued)

Species	Test conditions	Biological endpoint	LOEC (ng TBT/L)	Reported as[a]	Reference
Pseudodiaptomus marinus	Full life-cycle test	F_0 fecundity (nauplii/female)	60.00	nom	Huang et al. (2006)
Pseudodiaptomus marinus	Full life-cycle test	Sex ratio F_1	20.00	nom	Huang et al. (2006)
Pseudodiaptomus marinus	Full life-cycle test	Ovigerous F_0 females (%)	60.00	nom	Huang et al. (2006)
Schmackeria poplesia	Full life-cycle test	Ovigerous females (%)	10.00	nom	Huang et al. (2010)
Schmackeria poplesia	Full life-cycle test	Larval development	60.00	nom	Huang et al. (2010)
Tigriopus japonicus	Ovigerous females, 14 days	Nauplii production	50.00	nom	Ara et al. (2010)
Molluscs					
Biomphalaria glabrata	From hatchlings to adults, prolonged exposure	Egg laying	1.00	nom	Ritchie et al. (2005)
Crassostrea gigas	Oyster spats, 28 days	Growth (weight gain)	5.00	nom?	Nell and Chvojka (1992)
Lymnaea stagnalis	Adults, 21 days	Polyembryony	46.36	mm	Giusti et al. (2013b)
Lymnaea stagnalis	Adults, 56 days	Fecundity	372.82	gmm	Charles et al. (2016)
Lymnaea stagnalis	Adults, 21 days	Growth (shell size)	481.47	mm	Giusti et al. (2013b)
Lymnaea stagnalis	Eggs, 21 days	Mean hatching time	100.00	nom	Bandow and Weltje (2012)
Lymnaea stagnalis	Adults, 170 days	Fecundity	1000.00	nom	Leung et al. (2007)
Nucella lapillus	Egg capsule to adults, 1 year	Reproduction	27.80	mm	Harding et al. (2003)
Marisa cornuarietis	Adults, 8 weeks	Embryo production	39.50	nom	Schulte-Oehlmann (1997)
Mercenaria mercenaria	Veliger larvae, 14 days static renewal	Growth (shell size)	5.00	nom	Laughlin et al. (1988)
Mytilus edulis	Larvae, 33 days	Growth (shell size)	50.00	nom	Lapota et al. (1993)

Potamopyrgus antipodarum	Adults, 28 days	Fecundity	184.71	mm	Ruppert et al. (2016)
Pinctada fucata martensii	One-week static renewal exposure of adult females	Embryo development	191.00	mm	Inoue et al. (2004)
Pinctada fucata martensii	24-h static exposure of fertilized eggs	Embryo development	192.00	mm	Inoue et al. (2004)
Ruditapes philippinarum	24-h static exposure of fertilized eggs	Embryo development	62.00	mm	Inoue et al. (2006)
Saccostrea commercialis	Oyster spats, 28 days	Growth (weight gain)	5.00	nom?	Nell and Chvojka (1992)
Scrobicularia plana	30-day static renewal exposure of 10-day old pediveliger larvae	Larval shell growth	30.00	mm	Ruiz et al. (1995)
Echinoderms					
Paracentrotus lividus	fertilized eggs, 48 h	Embryo development and larval length	200.00	nom	Bellas et al. (2005))
Ascidians					
Ciona intestinalis	Embryos (2-cell stage), 20 h	Embryo development	4000.00	nom	Bellas et al. (2005)
Ciona intestinalis	Exposure of oocytes and spermatozoa	Fertilization rate	290,000.00	nom	Gallo and Tosti (2013)

[a]*nom* nominal, *mm* arithmetic mean measured, *gmm* geometric mean measured
For each species, the lowest LOEC value was used to construct the SSD (Fig. 4)

Table 3 NOEC (no observed effect concentration) of TBT for population-relevant endpoints in aquatic organisms

Species	Test duration	Biological endpoint	NOEC (ng TBT/L)	Reported as[a]	Reference
Fish					
Cyprinodon variegatus	Juveniles, 24 days	Growth	340.00	nom?	De Bruijn et al. (2005)
Danio rerio	Full life-cycle test	Sperm abnormality 3–5 months post-exposure	0.01	nom	McAllister and Kime (2003)
Danio rerio	Larvae, 0–70 days post-hatch	Sex ratio	0.01	nom	McAllister and Kime (2003)
Fundulus heteroclitus	Full life-cycle test	Sex ratio F_1	70.00	mm	Mochida et al. (2010)
Fundulus heteroclitus	Full life-cycle test	Hatchability F_0 (%)	750.00	mm	Mochida et al. (2010)
Fundulus heteroclitus	Full life-cycle test	Time to hatch F_1	520.00	mm	Mochida et al. (2010)
Gasterosteus aculeatus	225 days	Reproduction	100.00	mm?	De Bruijn et al. (2005)
Oncorhynchus mykiss	Juveniles, 16 weeks	Growth	60.00	nom?	De Bruijn et al. (2005)
Pimephales promelas	Early life-stage test, 32 days	Fry growth and weight	150.00	mm?	Brooke et al. (2003)
Poecilia reticulata	Juveniles, 91 days	Growth	320.00	nom?	De Bruijn et al. (2005)
Sebastiscus marmoratus	Embryos (gastrula stage), 144 h	Hatchability	1.00	nom	Zhang et al. (2011)
Crustaceans					
Acanthomysis sculpta	Life-cycle test	Reproduction	90.00	mm	Davidson et al. (2003)
Acartia tonsa	Eggs, 8 days	Larval development EC_{10}	0.70	nom	Kusk and Petersen (1997)
Caprella danilevskii	Full life-cycle test	Sex ratio	10.00	nom	Ohji et al. (2002)
Daphnia magna	Adults, 21 days	Reproduction	160.00	mm?	Kühn et al. (2005)
Daphnia magna	Adults, 21 days	Offspring/female	1250.00	nom	Oberdorster et al. (1998)

Eurytemora affinis	Adults, 13 days	Reproduction	10.00	mm?	De Bruijn et al. (2005)
Eurytemora affinis	Egg-carrying females, 13 days	Brood size	224.00	mm	Hall et al. (2003)
Pseudodiaptomus marinus	Full life-cycle test	Sex ratio F_1	6.00	nom	Huang et al. (2006)
Pseudodiaptomus marinus	Full life-cycle test	Ovigerous F_0 females (%)	20.00	nom	Huang et al. (2006)
Pseudodiaptomus marinus	Full life-cycle test	F_0 fecundity (nauplii/female)	20.00	nom	Huang et al. (2006)
Schmackeria poplesia	Full life-cycle test	Ovigerous females (%)	5.00	nom	Huang et al. (2010)
Schmackeria poplesia	Full life-cycle test	Larval development	40.00	nom	Huang et al. (2010)
Tigriopus japonicus	Ovigerous females, 14 days	Nauplii production	25.00	nom	Ara et al. (2010)
Molluscs					
Isognomon californicum	Gametes 48 h	Fertilization rate	1000.00	nom	Ringwood (1992)
Isognomon californicum	Embryos, 48 h	Embryo development	100.00	nom	Ringwood (1992)
Isognomon californicum	Veliger larvae, 4 days	Larval growth	20.00	nom	Ringwood (1992)
Lymnaea stagnalis	Adults, 170 days	Fecundity	10.00	nom	Leung et al. (2007)
Lymnaea stagnalis	Adults, 170 days	Population growth rate	2745.00	nom	Leung et al. (2007)
Lymnaea stagnalis	Adults, 56 days	Fecundity	231.00	gmm	Charles et al. (2016)
Lymnaea stagnalis	Adults, 21 days	Growth (shell size)	229.74	mm	Giusti et al. (2013a)
Lymnaea stagnalis	Eggs, 21 days	Mean hatching time	30.00	nom	Bandow and Weltje (2012)
Nucella lapillus	Egg capsule to adults, 1 year	Reproduction	7.40	mm	Harding et al. (2003)
Mytilus edulis	Larvae, 33 days	Growth (shell size)	6.00	nom	Lapota et al. (1993)
Pinctada fucata martensii	One-week static renewal exposure of adult females	Embryo development	92.00	mm	Inoue et al. (2004)

(continued)

Table 3 (continued)

Species	Test duration	Biological endpoint	NOEC (ng TBT/L)	Reported as[a]	Reference
Pinctada fucata martensii	24-h static exposure of fertilized eggs	Embryo development	91.00	mm	Inoue et al. (2004)
Potamopyrgus antipodarum	Adults, 28 days	Fecundity	95.65	mm	Ruppert et al. (2016)
Echinoderms					
Echinometra mathaei	Gametes, 60–90 min	Fertilization rate	1000.00	nom	Ringwood (1992)
Ophioderma brevispina	28 days	Regeneration	10.00	mm?	Walsh et al. (2005)
Paracentrotus lividus	Fertilized eggs, 48 h	Embryo development and larval length	100.00	nom	Bellas et al. (2005)
Ascidians					
Ciona intestinalis	Embryos (2-cell stage), 20 h	Embryo development	2000.00	nom	Bellas et al. (2005)
Annelids					
Neanthes arenaceodentata	Adults, 70 days	Growth	50.00	nom?	Moore et al. (2003)

[a]*nom* nominal, *mm* arithmetic mean measured, *gmm* geometric mean measured
For each species, the lowest NOEC value was used to construct the SSD (Fig. 5)

Table 4 LOEC (lowest observed effect concentration) of TBT for mortality in aquatic organisms

Species	Test duration	Biological endpoint	LOEC (ng TBT/L)	Reported as[a]	Reference
Amphibians					
Xenopus tropicalis	FETAX-like test, 48 h	Tadpole development	50.00	nom	Guo et al. (2010)
Fish					
Cyprinodon variegatus	n.r.	Parental survival	560.00	mm?	United States Environmental Protection Agency - US EPA (2008)
Oryzias latipes	Embryonic stages, 96 h	Embryo survival	41,500.00	mm?	Bentivegna and Piatkowski (1998)
Crustaceans					
Acartia tonsa	Nauplii larvae, 6 days	Larval survival	23.50	mm	Bushong et al. (1990)
Caprella danilevskii	Full life-cycle test	Embryo survival	10.00	nom	Ohji et al. (2003b)
Daphnia magna	21-day chronic	Survival and reproduction	200.00	mm?	Brooke et al. (2003))
Daphnia magna	21-day chronic	Survival and reproduction	340.00	mm?	ABC Laboratories Inc. (2003)
Daphnia magna	21-day chronic run over two generations	Adult survival	2225.00	nom	Oberdorster et al. (1998)
Eurytemora affinis	n.r.	Neonate survival	88.00	mm?	Hall et al. (2003)
Palaemon serratus	Zoe I stage larvae, 48 h	Larval survival	62,500.00	nom	Bellas et al. (2005)
Pseudodiaptomus marinus	Nauplii to copepodites, 13 days	Larvae to adult survival F_0	60.00	nom	Huang et al. (2006)
Schmackeria poplesia	Full life-cycle test	Adult survival	20.00	nom	Huang et al. (2010)
Tisbe biminiensis	Adult (7–10 day-old), 48 h	Adult survival	34,000.00	nom	Varella Motta da Costa et al. (2014)
Molluscs					
Lymnaea stagnalis	Adults, 170 days	Adult survival	1000.00	nom	Leung et al. (2007)
Lymnaea stagnalis	Adults, 170 days	Juvenile survival	1000.00	nom	Leung et al. (2007)

(continued)

Table 4 (continued)

Species	Test duration	Biological endpoint	LOEC (ng TBT/L)	Reported as[a]	Reference
Mytilus edulis	33 days	Larval survival	50.00	nom	Lapota et al. (1993)
Ruditapes philippinarum	Static renewal exposure of veliger larvae (D-larvae stage) for 13 days	Survival and development of veliger larvae	130.00	mm	Inoue et al. (2007)
Scrobicularia plana	30-day static renewal exposure of 10-day-old pediveliger larvae	Larval survival	56.10	mm	Ruiz et al. (1995)
Insects					
Chironomus riparius	Fourth instar larvae, 48 h	Larval survival	48.80	nom	Hahn and Schulz (2002)
Annelids					
Hydroides elegans	Early development (egg to juvenile)	Adult (post-spawning female) survival	10,000.00	nom	Lau et al. (2007)
Hydroides elegans	Early development (egg to juvenile)	Juvenile survival	1000.00	nom	Lau et al. (2007)
Neanthes arenaceodentata	Adults, 70 days	Adult survival	500.00	nom?	Moore et al. (2003)

[a]*nom* nominal, *mm* arithmetic mean measured, *gmm* geometric mean measured

For each species, the lowest LOEC value was used to construct the SSD (Fig. 7). Species for which LOEC values were higher than 1000 ng/L were not considered sensitive and were not included in the SSD

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